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4.	Harwerth	J. Biol. Chem. (1992) 267: 15160-15167
5.	Sarup	Growth Regulation (1991) 1: 72-82
6.	Hudziak	Mol. Cell. Biol. (1989) 9: 1165-1172
7.	Fendly	Cancer Res. (1990) 50: 1550-1558

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was also decreased in group B (P=0.015). Results presented show that both granulocyte and monocyte phagocytic functions are altered in breast cancer patients early in the disease process, and are not caused by potentially myelosuppressive therapy [5]. Differences in these functions, which existed at the time of diagnosis, seem to be related to the progression of the disease and therefore could be of prognostic value.

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A New Model for the Interaction of EGF-like Ligands With Their Receptors: the New One-Two

W.J. Gullick

OVEREXPRESSION or mutation of the type 1 growth factor receptors and their ligands occurs frequently in human tumours, and in some cases is associated with prognosis and response to treatment. A deeper understanding of their mechanisms of activation would be useful in the design of receptor inhibitors.

For some years, the accepted model for interaction of epidermal growth factor (EGF) with its receptor has been that one ligand binds to a receptor monomer, and that this is followed by receptor dimerisation, forming a complex of two receptors and two ligands (2:2). I wish to consider a new model in which a single molecule of EGF binds to a receptor dimer (1:2).

Such a complex is necessarily asymmetric as EGF itself is asymmetric. This, therefore, requires that one face of the ligand binds to one site in the first receptor (site A) and, through another part of its surface, to a different site in the second receptor (site B). Is there any reason to believe that this could occur? Inspection of the primary sequence of the extracellular domain of the EGF receptor suggests a possible explanation in that this is composed of two related sequences, each containing a region rich in cysteine residues associated with another sequence. Experiments from Schlessinger and colleagues have demonstrated that the more C-terminal (domain III) of the two cysteine rich regions is largely responsible for ligand binding [1], but that mutations in the second N-terminal equivalent region (domain I) also, but less fundamentally, affect binding [2]. It is possible, therefore, that ligand binding occurs first to site A (domain III) in a monomer with moderate affinity, and that this is followed by dimerisation with a second receptor, but with the EGF binding to site B (domain I), thereby forming the high affinity complex.

What is the evidence in favour of this model? Mutational analysis of EGF suggests that substitution of either residue 41 [3] or 47 [4] prevents receptor recognition. However, these side chains are on opposite faces of the ligand [5]. In the 1:2 model, this would be predicted, but it is less easy to reconcile with the 2:2 structure. Secondly, in the closely related type 1 receptors, HER2 and HER3, NDF/Heregulin can be chemically cross-linked to either receptor in a heterodimer, suggesting that the ligand is in close proximity to each receptor molecule [6]. A precedent for this 1:2 complex is provided by the asymmetric structure of one molecule of growth hormone bound to two receptor proteins [7].

The principle evidence in favour of the 2:2 model is the stoichiometry of ligand binding determined by Weber and associates [8]. These measurements, despite being carefully performed, are, however, sensitive to small variations in specific activity of the iodinated EGF, and the accuracy of measurements of protein concentration by comparative Coomassie blue staining and autoradiography. The structure suggested here leads to some predictions which can be tested by experiment, which may help to resolve the issue. Binding of one ligand to a receptor heterodimeric complex would not, for instance, allow binding of another ligand. For example, EGF binding to a heterodimer of EGFR and HER3 would prevent binding of NDF/Heregulin and *vice versa*. A speculation arising from this model is that it is possible that the five different ligands, known to bind to the EGF receptor and the alpha and beta families of NDF/Heregulin (which differ in their C-terminal parts of the EGF-like element), may be able to stabilise different combinations of type 1 receptor heterodimers. I believe therefore that, for the moment, results of experiments performed on this highly interactive system of receptors should now be considered in the light of both models.

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Vol. 9, No. 3

p185^{HER2} Monoclonal Antibody Has Antiproliferative Effects In Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor

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The *HER2*/c-*erbB*-2 gene encodes the epidermal growth factor receptorlike human homolog of the rat *neu* oncogene. Amplification of this gene in primary breast carcinomas has been shown to correlate with poor clinical prognosis for certain cancer patients. We show here that a monoclonal antibody directed against the extracellular domain of p185^{HER2} specifically inhibits the growth of breast tumor-derived cell lines overexpressing the *HER2*/c-*erbB*-2 gene product and prevents *HER2*/c-*erbB*-2-transformed NIH 3T3 cells from forming colonies in soft agar. Furthermore, resistance to the cytotoxic effect of tumor necrosis factor alpha, which has been shown to be a consequence of *HER2*/c-*erbB*-2 overexpression, is significantly reduced in the presence of this antibody.

HER2/c-*erbB*-2, the human homolog of the rat proto-oncogene *neu* (4, 34), encodes a 1,255-amino-acid glycoprotein with extensive homology to the human epidermal growth factor (EGF) receptor (4, 21, 33, 34, 42). The *HER2*/c-*erbB*-2 gene product, p185^{HER2}, has all of the structural features and many of the functional properties of subclass I growth factor receptors (reviewed in references 43 and 44), including cell surface location and an intrinsic tyrosine kinase activity. However, the ligand for this putative growth factor receptor has not yet been identified.

Amplification of the *HER2*/c-*erbB*-2 gene has been found in human salivary gland and gastric tumor-derived cell lines (13, 34), as well as in mammary gland carcinomas (21, 22, 40, 42). Slamon et al. (35) surveyed 189 primary breast adenocarcinomas and determined that the *HER2*/c-*erbB*-2 gene was amplified in about 30% of the cases. Most importantly, *HER2*/c-*erbB*-2 amplification was correlated with a negative prognosis and high probability of relapse. Similar although less frequent amplification of the *HER2*/c-*erbB*-2 gene has been reported for gastric and colon adenocarcinomas (45, 46). Experiments with NIH 3T3 cells also suggest a direct role for the overexpressed, structurally unaltered *HER2*/c-*erbB*-2 gene product p185^{HER2} in neoplastic transformation. High levels of *HER2*/c-*erbB*-2 gene expression attained by coamplification of the introduced gene with dihydrofolate reductase by methotrexate selection (18) or by using a strong promoter (6) was shown to transform NIH 3T3 fibroblasts. Only cells with high levels of p185^{HER2} are transformed, i.e., have an altered morphology, are anchorage independent, and will form tumors in athymic mice.

Overexpression of p185^{HER2} may, furthermore, contribute to malignant tumor development by allowing tumor cells to evade one component of the antitumor defenses of the body, the activated macrophage (17). Macrophages play an important role in immune surveillance against neoplastic growth in vivo (1, 2, 38), and Urban et al. (39) have shown that tumor cells made resistant to macrophages display enhanced tumorigenicity. Tumor necrosis factor alpha (TNF-α) has been shown to play a role in activated macrophage-mediated tumor cell killing in vitro (3, 11, 23, 29, 39). NIH 3T3 cells transformed by a transfected and amplified *HER2*/c-*erbB*-2 cDNA show increased resistance to the cytotoxic effects of activated macrophages or TNF-α in direct correlation with increased levels of p185^{HER2} expression. Furthermore, breast tumor cell lines with high levels of p185^{HER2} exhibit resistance to TNF-α. Resistance to host antitumor defenses could facilitate the escape of cells from a primary tumor to establish metastases at distant sites.

To further investigate the consequences of alteration in *HER2*/c-*erbB*-2 gene expression in mammary gland neoplasia and to facilitate investigation of the normal biological role of the *HER2*/c-*erbB*-2 gene product, we have prepared monoclonal antibodies against the extracellular domain of p185^{HER2}. One monoclonal antibody (4D5) was characterized in more detail and was shown to inhibit in vitro proliferation of human breast tumor cells overexpressing p185^{HER2} and, furthermore, to increase the sensitivity of these cells to the cytotoxic effects of TNF-α.

MATERIALS AND METHODS

Cells and cell culture. Human tumor cell lines were obtained from the American Type Culture Collection. The mouse fibroblast line NIH 3T3/HER2-3$_{400}$, expressing an amplified *HER2*/c-*erbB*-2 cDNA under simian virus 40 early promoter control, and the vector-transfected control cell line NIH 3T3/CVN have been described previously (18).

Cells were cultured in a 1:1 mixture of Dulbecco modified Eagle medium and Ham nutrient mixture F-12 supplemented with 2 mM glutamine, 100 u of penicillin per ml, 100 μg of streptomycin per ml, and 10% serum. Human tumor cell lines were cultured with fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.); NIH 3T3 derivatives were cultured with calf serum (Hyclone Laboratories, Inc., Logan, Utah.).

Immunization. Female BALB/c mice were immunized with NIH 3T3/HER2-3$_{400}$ cells expressing high levels of

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p185^{HER2}. The cells were washed once with phosphate-buffered saline (PBS) and detached from the plate with PBS containing 25 mM EDTA. After low-speed centrifugation, the cells were suspended in cold PBS (2×10^7 cells per ml). Each mouse was injected intraperitoneally with 0.5 ml of this cell suspension on weeks 0, 2, 5, and 7.

On weeks 9 and 13, 100 μl of a Triton X-100 membrane preparation of p185^{HER2}, partially purified by wheat germ agglutinin chromatography (700 μg of protein per ml) (25), was administered intraperitoneally. Three days before fusion, 100 μl of the enriched p185^{HER2} protein was administered intravenously.

Fusion and screening. Mice with high antibody titers as determined by immunoprecipitation of p185^{HER2} were sacrificed, and their splenocytes were fused as described previously (26). Spleen cells were mixed at a 4:1 ratio with the fusion partner, mouse myeloma cell line X63-Ag8.653 (20), in the presence of 50% polyethylene glycol 4000. Fused cells were plated at a density of 2×10^5 cells per well in 96-well microdilution plates. The hypoxanthine-azaserine (12) selection for hybridomas was begun 24 h later. Beginning at day 10 postfusion, supernatants from hybridoma-containing wells were tested for the presence of antibodies specific for p185^{HER2} by an enzyme-linked immunosorbent assay with the wheat germ agglutinin chromatography-purified p185^{HER2} preparation (28). Enzyme-linked immunosorbent assay-positive supernatants were confirmed by immunoprecipitation and cloned twice by limiting dilution.

Large quantities of specific monoclonal antibodies were produced by preparation of ascites fluid; antibodies were then purified on protein A-Sepharose columns (Fermentech, Inc., Edinburgh, Scotland) and stored sterile in PBS at 4°C.

Immunoprecipitations and antibodies. Cells were harvested by trypsinization, counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), and plated 24 h before being harvested for analysis of p185^{HER2} expression. Cells were lysed at 4°C with 0.8 ml of HNEG lysis buffer (18) per 100-mm plate. After 10 min, 1.6 ml of lysis dilution buffer (HNEG buffer with 1% bovine serum albumin and 0.1% Triton X-100) was added to each plate, and the extracts were clarified by centrifugation at 12,000 × *g* for 5 min.

Antibodies were added to the cell extracts and allowed to bind at 4°C for 2 to 4 h. Immune complexes were collected by adsorption to protein A-Sepharose beads for 20 min and washed three times with 1 ml of HNEG buffer–0.1% Triton X-100. Autophosphorylation reactions were carried out for 20 min at 4°C in 50 μl of HNEG wash buffer containing 5 mM $MnCl_2$ and 3 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol, Amersham Corp., Arlington Heights, Ill.). The autophosphorylation reaction conditions have been described previously (18). Proteins were separated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels and analyzed by autoradiography.

The polyclonal antibody, G-H2CT17, recognizing the carboxy-terminal 17 amino acids of p185^{HER2}, has been described previously (18). The anti-EGF receptor monoclonal antibody 108 (16) was provided by Joseph Schlessinger, Rorer Biotechnology, Inc.

Fluorescence-activated cell sorting. SK-BR-3 human breast tumor cells overexpressing the *HER2*/*c-erbB*-2 gene (17, 22) or A431 human squamous carcinoma cells overexpressing the EGF receptor gene (14) were grown in T175 flasks. They were detached from the flasks by treatment with 25 mM EDTA–0.15 M NaCl, collected by low-speed centrifugation, and suspended at 1×10^6 cells per ml in PBS–1% fetal bovine serum. One milliliter of each cell line was incubated with 10 μg of either anti-*HER2*/*c-erbB*-2 monoclonal antibody (4D5) or a control antibody (40.1.H1) recognizing the hepatitis B surface antigen. The cells were washed twice and suspended on ice for 30 min in 1 ml of PBS–1% fetal bovine serum containing 10 μg of goat anti-mouse immunoglobulin G $F(ab')_2$ fragments conjugated with fluorescein isothiocyanate dye (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Unbound fluorescein dye was removed by two further washes. The cells were suspended at 2×10^6 per ml in PBS–1% fetal bovine serum and analyzed with an EPICS 753 (Coulter) fluorescence-activated cell sorter. Fluorescein was excited by 300 mW of 488-nm argon laser light, and the emitted light was collected with a 525-nm band-pass filter with a 10-nm band width.

Down-regulation assay. SK-BR-3 cells were plated at 1.5×10^5 cells per 35-mm culture dish in normal medium. After a 6-h period to allow attachment, the medium was replaced by 1.5 ml of methionine-free labeling medium containing 150 μCi of [^{35}S]methionine per ml and 2% dialyzed fetal bovine serum. The cells were metabolically labeled for 14 h and then chased with medium containing 2% dialyzed serum and unlabeled methionine. Either a control monoclonal antibody (40.1.H1) or anti-p185^{HER2} (4D5) was added to a final concentration of 2.5 μg/ml. At 0, 5, and 11 h, extracts were prepared with 0.3 ml of lysis solution and 0.6 ml of dilution buffer. The p185^{HER2} was immunoprecipitated with 2.5 μl of polyclonal antibody G-H2CT17. The washed immune complexes were dissolved in sample buffer, electrophoresed on a SDS-7.5% polyacrylamide gel, and analyzed by autoradiography. Each time point determination was performed in duplicate. Autoradiograph band intensities were quantitated by using a scanner (Ambis Systems).

Cell proliferation assays. The anti-p185^{HER2} monoclonal antibodies were characterized by using the breast tumor cell line SK-BR-3. Cells were detached by using 0.25% (vol/vol) trypsin and suspended in complete medium at a density of 4

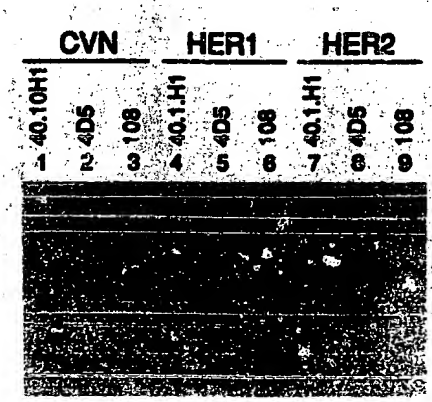

FIG. 1. Specificity of monoclonal antibody 4D5. Three cell lines, NIH 3T3/CVN, NIH 3T3/HER1-EGF receptor, and NIH 3T3/HER2-3_{400}, were plated out at 2.0×10^6 in 100-mm culture dishes. At 24 h, Triton X-100 lysates were prepared and divided into three portions. Either an irrelevant monoclonal antibody (6 μg of anti-hepatitis B virus surface antigen, 40.1.H1; lanes 1, 4, and 7), anti-p185^{HER} monoclonal antibody 4D5 (6 μg; lanes 2, 5, and 8), or anti-EGF receptor monoclonal antibody 108 (6 μg; lanes 3, 6, and 9) was added and allowed to bind at 4°C for 4 h. The immune complexes were collected with 30 μl of protein A-Sepharose. Rabbit anti-mouse immunoglobulin (7 μg) was added to each 4D5 immunoprecipitation to improve the binding of this monoclonal antibody to the protein A-coated beads. Proteins were labeled by autophosphorylation and separated on an SDS–7.5% polyacrylamide gel. The gel was exposed to film at −70°C for 4 h with an intensifying screen. The arrows show the positions of proteins of M_r 185,000 and 170,000.

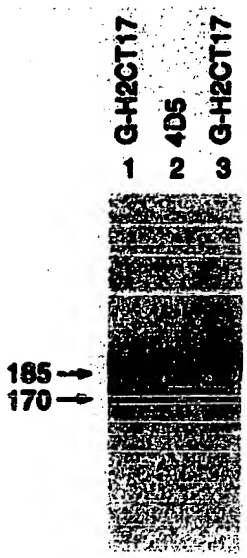

FIG. 2. Binding of monoclonal antibody 4D5 to unglycosylated receptor. NIH 3T3/HER2-3_{400} cells were plated into two 100-mm plates at 2×10^6 cells per plate. After 14 h, the antibiotic tunicamycin was added to one plate at 3 μg/ml. After a further 5.5 h of incubation, Triton X-100 lysates were then prepared from each plate. Immunoprecipitations, the autophosphorylation reaction, and SDS-polyacrylamide gel electrophoresis were performed as described in the legend to Fig. 1. Lanes: 1, tunicamycin-treated cell lysate (one-third of a plate) immunoprecipitated with 2.5 μl of a polyclonal antibody directed against the C terminus of p185^{HER2}; 2, tunicamycin-treated cell lysate (one-third of a plate) immunoprecipitated with 6 μg of 4D5; 3, untreated control lysate (one-third of a plate) immunoprecipitated with the polyclonal antibody. The arrows show the locations of proteins of M_r 185,000 and 170,000.

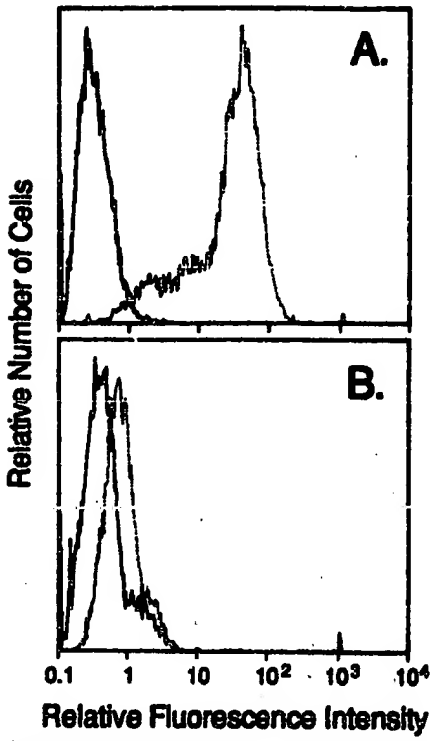

FIG. 3. Fluorescence-activated cell sorter histograms of human tumor cells binding anti-p185 monoclonal antibody 4D5. ——, Binding by the control antibody, 40.1.H1, directed against the hepatitis B surface antigen; ·····, binding by the anti-*HER2/c-erbB-2* antibody, 4D5. The antibodies were first allowed to react with the cell surface. After a wash step, bound antibody was labeled by addition of fluorescein-conjugated F(ab')$_2$ fragment of goat anti-mouse immunoglobulin G. (A) Binding of the antibodies to the human breast tumor line SK-BR-3, which contains an amplification of the *HER2/c-erbB-2* gene and expresses high levels of the *HER2/c-erbB-2* gene product p185^{HER2}. (B) Binding of the same antibodies to the human squamous epithelial cell line A431. This cell line expresses low levels of mRNA for *HER2/c-erbB-2* and high levels (2×10^6 receptors per cell) of the EGF receptor.

$\times 10^5$ cells per ml. Aliquots of 100 μl (4×10^4 cells) were plated into 96-well microdilution plates, the cells were allowed to adhere, and 100 μl of media alone or media containing monoclonal antibody (final concentration, 5 μg/ml) was then added. After 72 h, plates were washed twice with PBS (pH 7.5), stained with crystal violet (0.5% in methanol), and analyzed for relative cell proliferation as described previously (36).

For assays in which monoclonal antibodies were combined with recombinant human TNF-α (5.0×10^7 U/mg; Genentech, Inc.), cells were plated and allowed to adhere as described above. Following cell adherence, control medium alone or medium containing monoclonal antibodies was added to a final concentration of 5 μg/ml. Cultures were incubated for another 4 h, and then increasing concentrations of TNF-α were added to a final volume of 200 μl. Following 72 h of incubation, the relative cell number was determined by crystal violet staining. Some samples were analyzed by crystal violet staining following cell adherence for determination of the initial cell number.

RESULTS

Specificity of monoclonal antibody 4D5. Monoclonal antibodies directed against the extracellular domain of p185^{HER2} were prepared by immunizing mice with NIH 3T3 cells transfected with a *HER2/c-erbB-2* cDNA (HER2-3_{400}) (17, 18) and overexpressing the corresponding gene product, p185^{HER2}. One antibody exhibited several interesting biological properties and was chosen for further characterization. Antibody 4D5 specifically immunoprecipitated a single ^{32}P-labeled protein of M_r 185,000 from NIH 3T3 cells expressing p185^{HER2} (Fig. 1, lane 8). This antibody did not cross-react with the human EGF receptor (HER1; Fig. 1, lane 5), even when overexpressed in a mouse NIH 3T3 background (Fig. 1, lane 6). Furthermore, it did not immunoprecipitate any proteins from NIH 3T3 cells transfected with a control plasmid (pCVN) which expresses the neomycin resistance and dihydrofolate reductase genes only (Fig. 1, lane 2).

To determine the nature of the epitope recognized by 4D5, NIH 3T3/HER2-3_{400} cells were treated with tunicamycin, which prevents addition of N-linked oligosaccharides to proteins (15, 41). Cells treated with this antibiotic for 5.5 h contained two proteins which were immunoprecipitated by a polyclonal antibody against the carboxy-terminal peptide of p185^{HER2} (Fig. 2, lane 1). The polypeptide of 170,000 M_r represents unglycosylated p185^{HER2}. The upper band of ca. 185,000 M_r comigrated with glycosylated p185^{HER2} from untreated cells (Fig. 2, lane 3). Monoclonal antibody 4D5 efficiently immunoprecipitated only the glycosylated form of p185^{HER2} (Fig. 2, lane 2). This experiment suggests either that the epitope recognized by 4D5 consists partly of carbohydrate, or, alternatively, that the antibody recognizes a conformation of the protein achieved only when it is glycosylated.

TABLE 1. Inhibition f SK-BR-3 proliferation by anti-p185^{HER2} monoclonal antibodies[a]

Monoclonal antibody	Relative cell proliferation[b]
7C2	79.3 ± 2.2
2C4	79.5 ± 4.4
7D3	83.8 ± 5.9
4D5	44.2 ± 4.4
3E8	66.2 ± 2.4
6E9	98.9 ± 3.6
7F3	62.1 ± 1.4
3H4	66.5 ± 3.9
2H11	92.9 ± 4.8
40.1.H1	105.8 ± 3.8
4F4	94.7 ± 2.8

[a] SK-BR-3 breast tumor cells were plated as described in Materials and Methods. Following adherence, medium containing 5 μg of either anti-p185^{HER2} or control monoclonal antibodies (40.1.H1 and 4F4) per ml were added.

[b] Relative cell proliferation was determined by crystal violet staining of the monolayers after 72 h. Values are expressed as a percentage of results with untreated control cultures (100%).

The binding of monoclonal antibody 4D5 to human tumor cell lines was investigated by fluorescence-activated cell sorting (Fig. 3). This antibody was bound to the surface of cells expressing p185^{HER2}. Figure 3A shows the 160-fold increase in cellular fluorescence observed when 4D5 was added to SK-BR-3 breast adenocarcinoma cells relative to a control monoclonal antibody. This cell line contains an amplified *HER2*/c-*erbB*-2 gene and expresses high levels of p185^{HER2} (17, 22). In contrast, the squamous carcinoma cell line A431, which expresses about 2×10^6 EGF receptors per cell (14) but only low levels of p185^{HER2} (4), exhibited only a twofold increase in fluorescence with 4D5 (Fig. 3B) when compared with a control monoclonal antibody.

The binding of 4D5 correlated with the levels of p185^{HER2} expressed by these two cell lines. SK-BR-3 cells, expressing high levels of p185^{HER2}, showed an 80-fold increase in relative fluorescence intensity compared with A431 cells. This experiment demonstrates that 4D5 specifically recognizes the extracellular domain of p185^{HER2}.

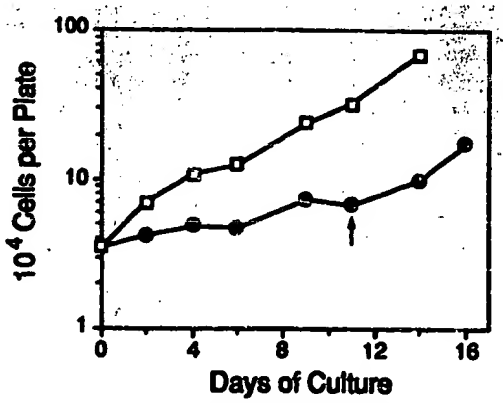

FIG. 4. Growth curve of SK-BR-3 cells treated with anti-*HER2*/c-*erbB*-2 monoclonal antibody 4D5. Cells were plated into 35-mm culture dishes at 20,000 cells per plate in medium containing 2.5 μg of either control antibody (40.1.H1, anti-hepatitis B surface antigen) (□) or anti-p185^{HER2} antibody 4D5 (●) per ml. On the indicated days, cells were trypsinized and counted in a Coulter counter. The determination for each time point and each antibody was done in duplicate, and the counts were averaged. The arrow indicates the day the cells were refed with medium without antibodies.

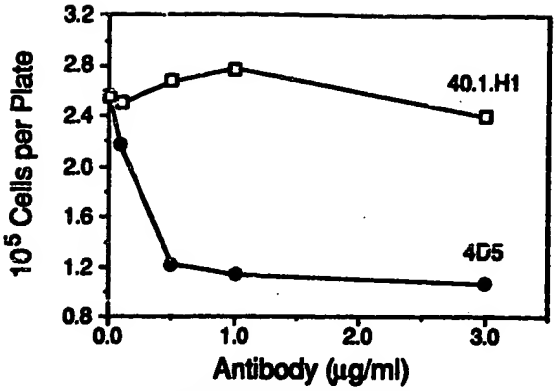

FIG. 5. Growth of SK-BR-3 cells in different concentrations of monoclonal antibody 4D5. The human breast tumor line SK-BR-3 was plated into 35-mm culture dishes at 20,000 cells per dish. Either 0.1, 0.5, 1.0, or 3.0 μg of a control monoclonal antibody (40.1.H1, anti-hepatitis B surface antigen) or monoclonal 4D5 antibody per ml was added at the time of plating. After 8 days of growth, the plates were trypsinized and the cells were counted in a Coulter counter. Each concentration of antibody was plated and counted in duplicate, and the cell numbers were averaged.

Effects on cell proliferation. We used the human mammary gland adenocarcinoma cell line, SK-BR-3, to determine whether monoclonal antibodies directed against the extracellular domain of p185^{HER2} had any effect on the proliferation of cell lines overexpressing this receptorlike protein. SK-BR-3 cells were coincubated with several *HER2*/c-*erbB*-2-specific monoclonal antibodies or with either of two different control monoclonal antibodies (40.1.H1, directed against the hepatitis B surface antigen; 4F4, directed against recombinant human gamma interferon). Most anti-*HER2*/c-*erbB*-2 monoclonal antibodies which recognize the extracellular domain inhibited the growth of SK-BR-3 cells (Table

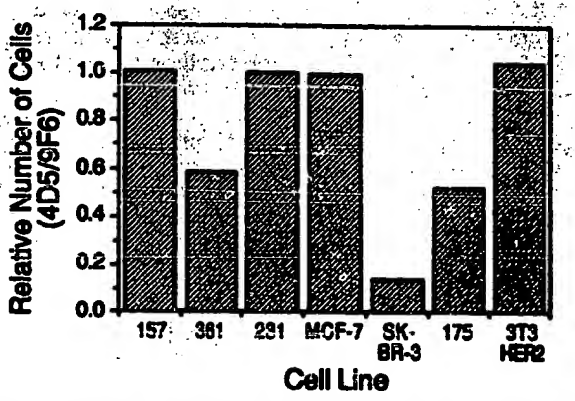

FIG. 6. Screening of breast tumor cell lines for growth inhibition by monoclonal antibody 4D5. Each cell line was plated in 35-mm culture dishes at 20,000 cells per dish. Either a control monoclonal antibody (9F6, anti-human immunodeficiency virus gp120) or the anti-p185^{HER2} monoclonal antibody 4D5 was added on day 0 to 2.5 μg/ml. Because the different cell lines grow at different rates, the cell lines NIH 3T3/HER2-3_{400} and SK-BR-3 were counted after 6 days, cell lines MDA-MB-157, MDA-MB-231, and MCF-7 were counted after 9 days, and cell lines MDA-MB-175VII and MDA-MB-361 were counted after 14 days. The difference in growth between cells treated with 4D5 and 40.1.H1 is expressed as the rati of cell numbers with 4D5 versus a control monoclonal antibody, 9F6. Each cell line was assayed in duplicate f r each antibody, and the counts were averaged.

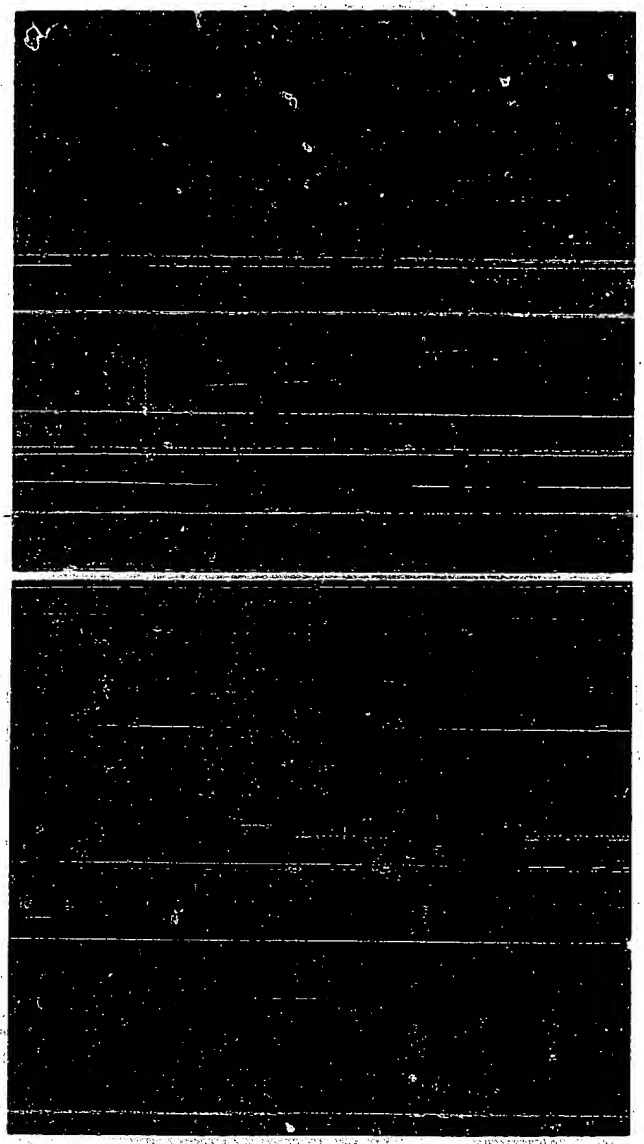

FIG. 7. Inhibition of anchorage-independent growth of NIH 3T3/HER2-3_{400} cells by 4D5. Cells (20,000 per 60-mm plate) were plated in 0.2% soft agar over a 0.4% agar base. After 3 weeks, the plates were photographed at ×100 magnification by using a Nikon microscope with phase-contrast optics. (a) HER2-3_{400} cells plated in agar containing 200 ng of a control antibody (TF-C8) per ml. (b) The same cells plated in agar containing 200 ng of 4D5 per ml.

1). Maximum inhibition was obtained with monoclonal antibody 4D5, which inhibited cellular proliferation by 56%. The control antibodies had no significant effect on cell growth.

Figure 4 compares the growth of SK-BR-3 cells in the presence of either a control antibody, 40.1.H1, or the anti-p185^{HER2} antibody. Proliferation of the cells was inhibited when antibody 4D5 was present. The generation time increased from 3.2 to 12.2 days. To determine whether 4D5 treatment was cytostatic or cytotoxic, antibody was removed by medium change 11 days after treatment. The cells resumed growth at a nearly normal rate, suggesting that the antibody affected cell growth rather than cell viability. The dose-response curve (Fig. 5) showed that a concentration of 200 ng/ml inhibited growth by 50%, whereas maximum

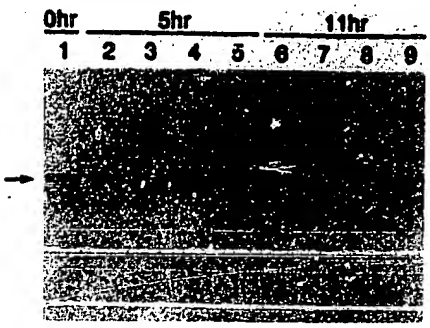

FIG. 8. Effect of antibody binding on p185^{HER2} turnover. SK-BR-3 cells were labeled for 14 h with [^{35}S]methionine. The label was then chased with cold methionine and either an irrelevant monoclonal antibody (40.1.H1, anti-hepatitis B surface antigen) or 4D5 was added to 2.5 μg/ml. The cells on the plates were lysed at 0, 5, and 11 h, and ^{35}S-labeled p185^{HER2} was quantitated by immunoprecipitation with the C-terminal specific polyclonal antibody. The 5- and 11-h time point determinations were performed in duplicate for each of the two antibodies. Proteins were separated by SDS-polyacrylamide gel electrophoresis. The fluor-treated gel was exposed to film for 4 h at room temperature. The arrow indicates the position of a protein of M_r 185,000. Band intensities were quantitated by using an Ambis Systems scanner. Lanes; 1, 0 h; lanes 2 and 3, 40.1.H1 (5 h); lanes 4 and 5, 4D5 (5 h); lanes 6 and 7, 40.1.H1 (11 h); lanes 8 and 9, 4D5 (11 h).

effects were achieved by using a concentration of between 0.5 and 1 μg/ml.

The effect of 4D5 on the proliferation of six additional breast tumor cell lines, as well as mouse NIH 3T3 fibroblasts transformed by p185^{HER2} overexpression (NIH 3T3/HER2-3_{400}), was tested in monolayer growth assays. Cells were plated at low density in medium containing 2.5 μg of either a control antibody or 4D5 per ml. When the cultures approached confluency, cells were removed with trypsin and counted. 4D5 did not have any significant effect on the growth of the MCF-7, MDA-MB-157, MDA-MB-231, or NIH 3T3/HER2-3_{400} cell lines (Fig. 6). It did, however, significantly affect the growth of the cell lines MDA-MB-361 (58% of control) and MDA-MB-175-VII (52% of control), which express high levels of p185^{HER2} (17).

Interestingly, monoclonal antibody 4D5 had no effect on the monolayer growth of the NIH 3T3/HER2-3_{400} cell line. However, it completely prevented colony formation by these cells in soft agar (Fig. 7), a property which had been induced by *HER2/c-erbB-2* amplification (18). In the presence of 200 ng of a control monoclonal antibody (antitissue factor, TC-C8) per ml, 116 (average of two plates) soft-agar colonies were counted, while the same cells plated simultaneously into soft agar containing 200 ng of 4D5 per ml did not yield any colonies.

Monoclonal antibody 4D5 down-regulates p185^{HER2}. To determine whether the antiproliferative effect of 4D5 was due to enhanced degradation of p185^{HER2}, we measured its rate of turnover in the presence or absence of antibody. p185^{HER2} was metabolically labeled by culturing SK-BR-3 cells for 14 h in the presence of [^{35}S]methionine. Cells were then chased for various times, and either a control antibody or 4D5 was added at the beginning of the chase period. At 0, 5, and 11 h, cells were lysed and p185^{HER2} levels were assayed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. p185^{HER2} is degraded more rapidly after exposure of SK-BR-3 cells to 4D5 (Fig. 8). Densitometric evaluation of the data showed that the p185^{HER2} half-life of

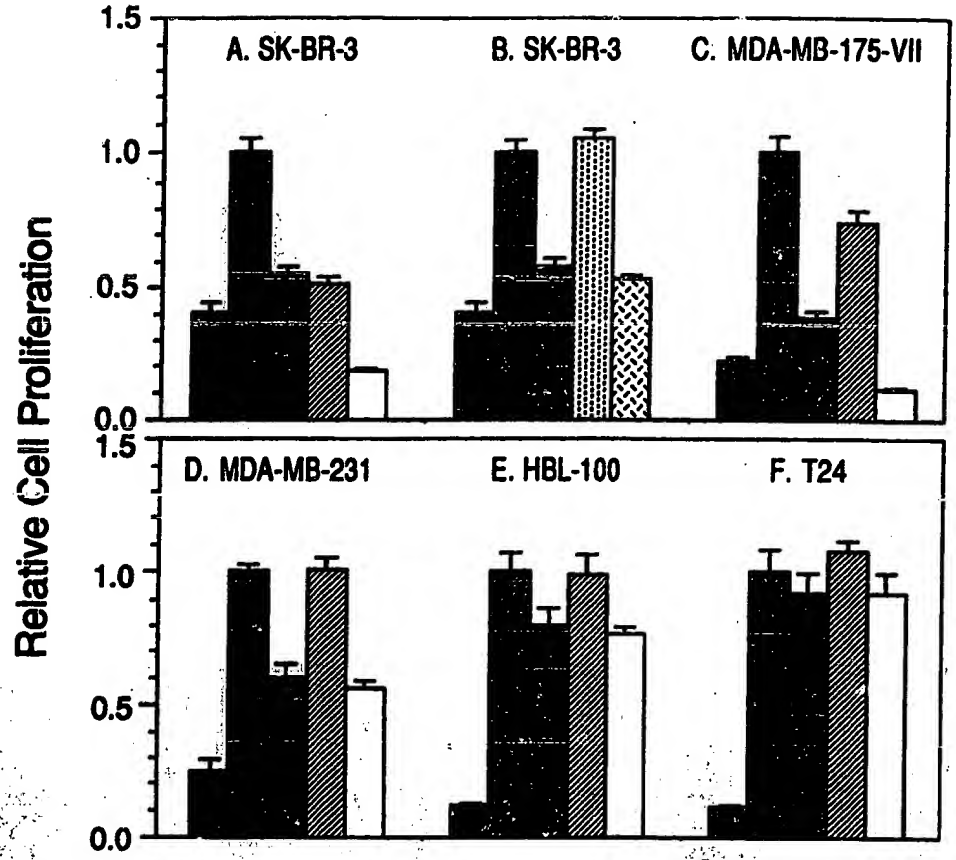

FIG. 9. Monoclonal antibody 4D5 sensitizes breast tumor cells to the cytotoxic effects of TNF-α. Cells were plated in 96-well microdilution plates (4×10^4 cells per well for SK-BR-3, MDA-MB-175-VII, and MDA-MB-231; 10^4 cells per well for HBL-100 and T24) and allowed to adhere for 2 h. Anti-*HER2*/*c-erbB*-2 monoclonal antibody 4D5 (5 μg/ml) or anti-hepatitis B surface antigen monoclonal antibody 40.1.H1 (5 μg/ml) was then added for a 4-h incubation prior to the addition of TNF-α to a final concentration of 10^4 units/ml. After 72 h, the monolayers were washed twice with PBS and stained with crystal violet dye for determination of relative cell proliferation. In addition, some cell monolayers were stained with crystal violet following adherence in order to determine the initial cell density for comparison with cell densities measured after 72 h. The symbols denote initial cell density (■), untreated (control) cells (■), cells treated with TNF-α (■), 4D5 (▨), TNF-α plus 4D5 (□), 40.1.H1 (⁞⁞⁞); or TNF-α plus 40.1.H1 (⋰⋰).

7 h decreased to 5 h in the presence of antibody (data not shown).

Monoclonal antibody 4D5 enhances TNF-α cytotoxicity. The addition of certain growth factors to tumor cells has been shown to increase their resistance to the cytotoxic effects of TNF-α (37). A prediction based on these findings would be that expression of oncogenes that mimic or replace growth factor receptor function may also increase the resistance of cells to this cytokine. Recently, it was shown that overexpression of the putative growth factor receptor $p185^{HER2}$ in NIH 3T3 cells caused an increase in the resistance of these cells to TNF-α (17). Furthermore, breast tumor cell lines with high levels of $p185^{HER2}$ also exhibited TNF-α resistance.

To further investigate the mechanism by which the 4D5 antibody inhibited cell growth, we investigated the response of three breast tumor cell lines to TNF-α in the presence or absence of this antibody. If the anti-$p185^{HER2}$ monoclonal antibody 4D5 inhibited proliferation of breast tumor cells by interfering with the signalling functions of $p185^{HER2}$, addition of this antibody would be expected to enhance the sensitivity of tumor cells to TNF-α. Both SK-BR-3 (Fig. 9A) and MDA-MB-175-VII (Fig. 9C) were growth inhibited by both the monoclonal antibody 4D5 (5 μg/ml; 50% and 25% inhibition, respectively) and high concentrations of TNF-α (1×10^4 units/ml; 50% and 60% inhibition, respectively). However, the combination of TNF-α and monoclonal antibody 4D5 reduced the SK-BR-3 and MDA-MB-175-VII tumor cell number to a level below that initially plated, indicating the induction of a cytotoxic response. In a separate experiment, SK-BR-3 cell viability was determined directly by using trypan blue dye exclusion, yielding identical results to those described above that were obtained by using crystal violet staining (data not shown). A control monoclonal antibody, 40.1.H1, did not inhibit SK-BR-3 breast tumor cell proliferation, nor did it induce an enhanced sensitivity of this cell line to the cytotoxic effects of TNF-α (Fig. 9B). In addition, the growth of the breast tumor cell line MDA-MB-231, which does not express detectable levels of $p185^{HER2}$ (17), was unaffected by monoclonal antibody 4D5, and the growth inhibition seen with the combination of 4D5 and TNF-α was similar to that observed with TNF-α alone (Fig. 9D). Furthermore, neither HBL-100 (30), a nontransformed but immortalized human breast epithelial cell line (Fig. 9E), nor T24 (27), a human bladder carcinoma cell line (Fig. 9F), expressed high levels of $p185^{HER2}$ (data not shown), and neither demonstrated growth inhibition by 4D5 or an enhanced growth-inhibitory or cytotoxic response to the combination of TNF-α and monoclonal antibody 4D5. These results demonstrate that only tumor cells which

overexpress p185^{HER2} will become sensitized to the cytotoxic effects of TNF-α by antibody 4D5.

DISCUSSION

We have prepared monoclonal antibodies against the extracellular domain of the *HER2*/c-*erbB*-2 gene product, p185^{HER2}, and have found that one of these, 4D5, strongly inhibits the growth of several breast tumor cell lines and furthermore sensitizes p185^{HER2}-overexpressing breast carcinoma cell lines SK-BR-3 and MDA-MB-175-VII to the cytotoxic effects of TNF-α. Monoclonal antibody 4D5 is specific for p185^{HER2} and shows no cross-reactivity with the closely related human EGF receptor expressed in mouse fibroblasts. Of six mammary carcinoma cell lines tested, only the three lines which express high levels of p185^{HER2} (SK-BR3, MBA-MB-175, and MDA-MD-361 [17]) were growth inhibited, and 4D5 did not inhibit the proliferation of a nontransformed human breast epithelial cell line, HBL-100, or the bladder carcinoma cell line T24.

In the presence of the antibody, the inhibition of SK-BR-3 cell growth was nearly complete, but the effect was cytostatic rather than cytotoxic. This property of 4D5 is similar to that described for a subset of monoclonal antibodies to the EGF receptor (19, 31, 32) which inhibit the growth of A431 cells, a human squamous epithelial carcinoma line expressing high levels of the EGF receptor. In this case, these inhibitory antibodies compete with radiolabeled EGF for binding to the receptor, and antibodies which do not block EGF binding have no effect on A431 cell growth. It has been suggested (J. Mendelsohn and H. Masui, Clin. Res. 35:600A, 1987) that these antibodies inhibit cell growth by interfering with an autocrine system involving the EGF receptor and an essential growth factor, transforming growth factor alpha, that is produced by the cells (5). It is therefore intriguing to speculate that antibody 4D5 analogously interferes with ligand binding to the *HER2*/c-*erbB*-2 gene product. Since an appropriate ligand for the putative *HER2*/c-*erbB*-2 receptor has not yet been identified, this possibility cannot yet be tested directly.

The 4D5 antibody had no effect on the growth of NIH 3T3 cells transformed by *HER2*/c-*erbB*-2 overexpression. However, it reversed one property conferred on these cells by amplification of the *HER2*/c-*erbB*-2 cDNA: the formation of colonies in soft agar was prevented by 200 ng of 4D5 antibody per ml. This result is similar to those obtained by Drebin et al. (8) with a monoclonal antibody to the rat *neu* oncogene-encoded p185^{neu}. They also observed that an anti-p185^{neu} monoclonal antibody inhibited colony growth in soft agar and tumor formation by *neu*-transformed NIH 3T3 cells in athymic mice (7–10). This effect was attributed to a lowering p185^{neu} levels by an increase in receptor turnover triggered by antibody binding. The apparent discrepancy between 4D5 effects on proliferation of breast tumor cells versus transfected mouse fibroblast cells is most probably a reflection of the fact that SK-BR-3 cells are authentic cancer cells, in contrast to the NIH 3T3 model system. Whereas SK-BR-3 cells may have evolved to be dependent on *HER2*/c-*erbB*-2-mediated signals for both growth and transformation characteristics, NIH 3T3 cells have acquired a transformed phenotype only as a result of *HER2*/c-*erbB*-2 overexpression, but may proliferate normally in response to other serum growth factors, even in the presence of blocking anti-p185^{HER2} antibody.

Previous work has shown that high-level expression of p185^{HER2} will transform NIH 3T3 cells and has suggested a casual role for amplification of the *HER2*/c-*erbB*-2 gene in mammary gland neoplasia. We have shown here that *HER2*/c-*erbB*-2 gene overexpression in NIH 3T3 cells is associated with increased resistance to the monokine TNF-α and that breast tumor cell lines which overexpress p185^{HER2} are resistant to the cytotoxic effects of TNF-α. The mechanism by which 4D5 inhibits breast tumor cell proliferation and reverses phenotypes associated with high levels of p185^{HER2} expression, such as resistance to TNF-α, is not clear. However, these results suggest that in addition to its ability to transform cells by virtue of overexpression (6, 18), *HER2*/c-*erbB*-2 could play a role in tumor progression by allowing tumor cells overexpressing p185^{HER2} to evade one component of the antitumor immunosurveillance of the host, the activated macrophage (17). These properties of the *HER2*/c-*erbB*-2 gene product may in part explain the aggressive, single-step induction of mammary adenocarcinoma in transgenic mice bearing the *neu* oncogene (24), which encodes the mutated rat homolog of p185^{HER2}.

The experiments presented here demonstrate that a monoclonal antibody which recognizes the extracellular domain of p185^{HER2} inhibits the proliferation of breast tumor cells which overexpress this receptorlike protein. Moreover, treatment with this antibody also sensitizes these tumor cells to the cytotoxic effects of TNF-α. Monoclonal antibodies specific for p185^{HER2} may therefore be useful therapeutic agents for the treatment of human neoplasias, including certain mammary carcinomas, which are characterized by the overexpressing of p185^{HER2}.

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Characterization of Murine Monoclonal Antibodies Reactive to Either the Human Epidermal Growth Factor Receptor or HER2/*neu* Gene Product

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ABSTRACT

High levels of expression of either the epidermal growth factor receptor or the receptor-like HER2/*neu* gene product p185^{HER2} have been observed in a variety of human malignancies. Because of the association of this high level expression with certain human tumors, we have generated a panel of monoclonal antibodies specific for either the epidermal growth factor receptor or p185^{HER2} to study their structure, function, and antigenic domains in the normal and neoplastic states. We used the epidermoid carcinoma line A431 to generate five monoclonal antibodies which immunoprecipitate the epidermal growth factor receptor. These monoclonal antibodies bind to the extracellular domain of the epidermal growth factor receptor and demonstrate variable effects on epidermal growth factor binding. We used a stably transfected NIH 3T3 cell line expressing the HER2/*neu* gene to produce and characterize 10 monoclonal antibodies which immunoprecipitate p185^{HER2}. These monoclonal antibodies bind to the extracellular domain of p185^{HER2} and do not cross-react with the epidermal growth factor receptor. The characteristics and potential applications of these monoclonal antibodies will be discussed.

INTRODUCTION

Alterations in growth factor receptor structure and level of expression have been implicated in the process of abnormal growth control, transformation, and oncogenesis. Some of these receptors share homology with retroviral oncogenes, have intrinsic tyrosine kinase activity, and are generally referred to as protooncogenes (for review see Ref. 1). Included in this group of receptors is the epidermal growth factor receptor which is the cellular homologue of the avian retrovirus oncogene v-*erb*B. The p185^{HER2} product[2] of the human EGFR related gene (HER2/*neu*) is a receptor-like tyrosine kinase without any oncogene homologue identified in a retrovirus. The EGFR and p185^{HER2} are similar but distinct glycoproteins encoded by genes located on human chromosomes 7 (2, 3) and 17 (4), respectively. The EGFR and p185^{HER2} have approximately 40% homology in their extracellular ligand binding domains and 78% homology in their intracellular ATP binding and tyrosine kinase regions as determined by their predicted primary amino acid sequences (4). The EGFR is a M_r 170,000 single chain glycoprotein of 1,186 amino acids with a 621-amino acid, extracellular, NH_2-terminal domain (5, 6). This extracellular domain of the EGFR binds the 53-amino acid, M_r 6,025 EGF (7) as well as the 50-amino acid, M_r 6,000 transforming growth factor α (8). In both cases binding of the growth factor results in activation of the receptor associated tyrosine kinase (9, 10). The p185^{HER2} receptor is also a single chain, M_r 185,000 glycoprotein of 1,255 amino acids with a 632-amino acid, extracellular domain which is thought to bind an unknown ligand (1, 4).

Amplification of expression of the EGFR is associated with human cervical and ovarian carcinomas (11), epidermal squamous cell carcinomas (12), and malignant gliomas (13). Amplification of the HER2/*neu* gene has been found in mammary gland carcinomas (14–16), and ovarian cancer (17) and is associated with the most aggressive forms of breast and ovarian tumors and poor prognosis from these cancers (17, 18). In addition, amplification of HER2/*neu* has also been shown in other human adenocarcinomas including salivary gland (19), stomach, and kidney (20). The MAbs to these cell surface glycoproteins and MAbs which are specific to phosphorylated tyrosines were generated to investigate expression of these protooncogenes in tumor cell lines and to study the structure/function relationships and antigenic domains of these receptors. We performed extensive specificity studies on the MAbs raised to either the EGFR or p185^{HER2} to evaluate shared antigenic sites on these related structures since these MAbs are candidates for the development of receptor based tumor therapies, *in vivo* and *in vitro* diagnostics, as well as important reagents for the development of assays to monitor the *in vitro* effects of receptor modulation.

MATERIALS AND METHODS

Cell Lines and Isolation of Receptors from Cells. The human epidermoid carcinoma A431 cell line (21) was used for isolation of the EGFR (22), since this cell line has been shown to express $2–3 \times 10^6$ copies/cell of the EGFR (21, 23–25). A431 cells were grown to confluence at 37°C and 10% CO_2 atmosphere in medium consisting of DMEM/Ham's F-12 (50/50, v/v), 5% fetal calf serum, 15 mM HEPES (pH 7.2), 50 units/ml penicillin, and 10 μg/ml streptomycin. The cells were solubilized in 1% Triton X-100 (26), and the EGFR was partially purified by WGA affinity chromatography. Elution of the receptor was achieved with 40 mM HEPES, pH 7.5, containing 0.3 M *N*-acetylglucosamine, 0.15 M NaCl, 0.2% Triton X-100, and 10% glycerol. Protein concentration was determined by the method of Bradford (27). The affinity (approximately 20 nM) of the solubilized receptor and the receptor concentration (approximately 5 pmol receptor/mg protein) were determined by Scatchard analysis of ^{125}I-EGF binding (28). EGF (Sigma, St. Louis, MO) was iodinated by the standard chloramine-T method (29). Tyrosine kinase activity was measured in the presence of 80 μCi/ml [γ-^{32}P]ATP, 10 μM ATP, 4 mM $MgCl_2$, in 2 mM $MnCl_2$ in 20 mM HEPES, pH 7.5, containing 0.1% Triton X-100, and 10% glycerol. HER2/*neu* transfected NIH 3T3 cells, NIH 3T3/HER2-3$_{400}$ (30), expressing approximately 1×10^5 p185^{HER2} molecules/cell (data not shown) were grown to confluency in the above media. The cells were extracted with 1% Triton X-100 and the membrane protein p185^{HER2} was partially purified by WGA affinity chromatography as described above. The tyrosine kinase activity was measured as above except the concentration of [γ-^{32}P]ATP was 100 μCi/ml and there was no unlabeled ATP in the kinase solution.

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[2] The abbreviations used are: p185, M_r 185,000 protein; gp120, M_r 120,00 glycoprotein (other proteins and glycoproteins similarly designated are); EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; MAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; WGA, wheat germ agglutinin-Sepharose; PBS, phosphate buffered saline, pH 7.2; ELISA, enzyme linked immunosorbent assay; PBST, PBS-0.05% Tween 20; HTG, 20 mM HEPES-0.1% Triton X-100-10% glycerol; RAM-SPA, protein A-Sepharose coated with rabbit anti-mouse IgG; SDS, sodium dodecyl sulfate; HNEG, 20 mM Hepes-150 mM NaCl-1 mM ethyleneglycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 10% glycerol; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; FACS, fluorescence activated cell sorter; RMF, relative mean fluorescence.

Generation of Monoclonal Antibodies Specific for the EGFR. BALB/c mice were immunized with 2–4 × 10^6 A431 cells in PBS, i.p. on weeks 0, 2, 4, and 6. The immunized mice were tested for an antibody response by immunoprecipitation of ^{32}P-labeled EGFR. The mice with the highest serum titers were given i.v. injections of a WGA purified A431 membrane extract during week 18. Three days later their splenocytes were fused with mouse myeloma line X63-Ag8.653 (31), using 50% polyethylene glycol 4000 by the procedure of Oi and Herzenberg (32). Fused cells were plated at a density of 2 × 10^5 cells/well in 96-well microtiter plates (day 0) and hybridoma selection was begun on day 1 with 10% fetal calf serum in DMEM media containing hypoxanthine/azaserine (33). Beginning on day 10 hybridoma supernatants were screened for the presence of EGFR specific antibodies as described below.

Generation of Monoclonal Antibodies Specific for p185^{HER2}. NIH 3T3/HER2-3_{400} cells were harvested with PBS containing 25 mM EDTA and were used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks 0, 2, 5, and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled p185^{HER2} were given i.p. injections of a WGA purified p185^{HER2} membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the p185^{HER2} preparation and the splenocytes were fused as described for the anti-EGFR fusions. The hybridoma supernatants were screened by ELISA as described below.

Generation of Monoclonal Antibodies Specific for Phosphotyrosine. BALB/c mice were immunized s.c. with 200 μg of phosphotyramine coupled to keyhole limpet hemocyanin by using a bromoacetyl linker (34) in Freund's adjuvant on weeks 0, 2, 4, and 6. The mouse with positive antisera that immunoprecipitated ^{32}P-phosphorylated EGFR most efficiently was immunized i.v. with phosphotyramine keyhole limpet hemocyanin in PBS on week 8 and the splenocytes were fused as described above. The initial screen was an ELISA on phosphotyramine coupled to soybean trypsin inhibitor. ELISA positive parental culture supernatants were further assayed by immunoprecipitation with ^{32}P-phosphorylated EGFR as described below.

Enzyme-linked Immunosorbent Assays. ELISAs were performed as initial screens of fusions, to monitor the purification of the anti-p185^{HER2} and anti-EGFR MAbs, initial screens of the phosphotyramine fusions, and as one approach to determine the cross-reactivity between anti-EGFR and anti-p185^{HER2} MAbs. Immuno II (NUNC Roskilde, Denmark) microtiter plates were coated with 1 μg/ml of either the WGA affinity purified p185^{HER2}, WGA affinity purified EGFR membrane preparations, or phosphotyramine coupled to soybean trypsin inhibitor in PBS overnight at 4°C or for 2 h at room temperature. Antigen coated plates were washed with PBST and MAb samples were incubated for 1–2 h at room temperature on a shaker. Plates were washed with PBST and goat anti-mouse IgG conjugated with horseradish peroxidase (Tago, Burlingame, CA) was added and incubated for 1 h. The plates were washed with PBST and 100 μl/well of *o*-phenylenediamine dihydrochloride substrate (Sigma) were added at 0.5 mg/ml in 0.05 M citrate/phosphate buffer. The reaction was stopped after 15–30 min at room temperature with 100 μl/well of 2.5 M H_2SO_4 and absorbance was determined at 492 nm on a multiscan plate reader (Flow, McLean, VA).

Purification of MAb from Ascites. Selected ELISA positive hybridomas were cloned twice by limiting dilution and ascites fluid containing specific MAb was produced in pristane primed BALB/c mice (35). Antibodies were purified from the ascites fluid by protein A-Sepharose (Fermentech, Inc., Edinburgh, Scotland), using established procedures (36), stored sterile in PBS at 4°C, and further characterized in a variety of immunoassays.

Radioimmunoprecipitation of [γ-^{32}P]ATP Phosphorylated Receptors. Radioimmunoprecipitations were performed by first autophosphorylating the appropriate Triton X-100 cell lysates with [γ-^{32}P]ATP in kinase buffer containing 180 μCi/ml [γ^{32}P]ATP, 5 μM ATP, 2 mM $MnCl_2$, 4 mM $MgCl_2$, and the WGA purified extracts of either the p185^{HER2} (35 μg/ml) or the EGFR (17.5 μg/ml) in HTG buffer for 30 min at room temperature. The kinase mixture, 50 μl, was then mixed with 2 μg of the appropriate MAb, diluted to 50 μl with HTG buffer, and incubated for 1 h at room temperature. Protein A-Sepharose CL-4B, 50 μl, (Pharmacia, Uppsala Sweden) coated with 1 mg/ml rabbit anti-mouse IgG to enhance binding of all IgG subclasses, was added and incubated for 1 h at room temperature. Samples were washed twice with HTG and once with PBS containing 0.2% deoxycholate and 0.2% Tween 20. Reducing SDS sample buffer containing 30 μM β-mercaptoethanol-2% SDS in 14 mM Tris, pH 6.8, 50 μl, was added to the pelleted immunocomplexes and the samples were heated to 90°C for 5 min. The RAM-SPA was separated by centrifuging for 2 min at 10,000 × *g* and the supernatant was loaded on a 7.5% SDS polyacrylamide gel (37), electrophoresed at constant 30-mA current and followed by autoradiography to determine the relative molecular weights of the bound autophosphorylated proteins.

Radioimmunoprecipitation of [^{35}S]Cysteine Labeled Receptors. Subconfluent cell cultures in 100-mm plates were washed once with cysteine-free DMEM and incubated overnight in 2 ml of cysteine-free media containing 10% dialyzed fetal bovine serum and 0.5 mCi [^{35}S]-cysteine (Amersham, Arlington, IL). The labeling medium was aspirated and the cells were rinsed with HNEG buffer at pH 7.6. The cells were lysed with 0.5 ml of HNEG buffer with 1% Triton X-100 and 1.5 mM $MgCl_2$ (lysis buffer), diluted with 1 ml HNEG and 1% BSA (dilution buffer), and the cell membrane fragments were removed by centrifugation at 10,000 × *g*. The labeled cell extract was preincubated with RAM-SPA for 30 min at room temperature and centrifuged as above to remove labeled proteins which bound nonspecifically. This preabsorbed extract was divided into 4–6 samples and incubated with 2 μg of the appropriate MAb for 30–60 min. The washing, immunoprecipitation, and electrophoresis steps were performed as described above.

Radioimmunoprecipitation of Nonglycosylated Receptors. Cells were harvested by trypsinization, counted (Coulter Counter), and plated into 150-mm culture dishes. A431 and NIH 3T3/HER2-3_{400} cells were plated at 2 × 10^6 or 6 × 10^6 cells/dish, respectively, and, after a 3-h attachment period, the antibiotic tunicamycin at 3 μg/ml was added to the experimental plates. Cell lysates were prepared after a further 6-h incubation and the anti-EGFR and the anti-p185^{HER2} MAb immunoprecipitations were [γ-^{32}P]ATP labeled by autophosphorylation at 4°C, washed, electrophoresed, and analyzed by autoradiography as described above. The polyclonal rabbit antibody, G-H2CT17, raised against the carboxy terminal 17 amino acids of p185^{HER2} (38), was used as a control antiserum to immunoprecipitate p185^{HER2} irrespective of glycosylation. Similarly, the polyclonal mouse antibody, R1/1080, raised to the EGFR, was used as the positive control for immunoprecipitation of the EGFR irrespective of glycosylation.

Isotyping of MAbs. Isotyping of the MAbs was performed by using the Pandex Screen Machine and anti-mouse IgG coated particles. Polystyrene Pandex assay particles, 1 ml of a 5% solution, were incubated with 1.0 mg of affinity purified rat anti-mouse IgG (PelFreez, Rogers, AZ) in carbonate buffer at pH 9.6 for 1 h at room temperature. The beads were washed twice with PBS and resuspended in 20 ml of PBS at 0.25% particles. Coated particles, 20 μl, were added to 20 μl of the appropriate MAb supernatants in wells of Pandex microtiter plates and incubated on a shaker at room temperature for 30 min. The plates were washed and FITC-conjugated rat monoclonal anti-mouse isotype specific reagents (generous gift of David Buck, Becton-Dickinson Monoclonal Center) were added at 1–2 μg/ml in PBST. Samples were incubated 15 min, washed, and the fluorescence was quantitated by the Pandex software.

Epitope Determination by MAb Competitive Binding Analysis. Cross-blocking studies were done on both panels of MAbs by direct fluorescence on intact cells by using the Pandex Screen Machine to quantitate fluorescence. Each MAb was conjugated with FITC, using established procedures (39). Confluent monolayers of A431 or NIH 3T3/HER2-3_{400} cells were trypsinized, washed once, and resuspended at 1.75 × 10^6 cell/ml in cold PBS containing 0.5% BSA (BSA/PBS) and 0.1% NaN_3. A final concentration of 1% latex particles (IDC, Portland, OR) was added to reduce clogging of the Pandex plate membranes. Cells in suspension, 20 μl, and 20 μl of purified MAbs (100 μg/ml to 0.1 μg/ml) were added to the Pandex plate wells and incubated on ice for 30 min. A predetermined dilution of FITC-labeled MAbs in 20 μl was

added to each well, incubated for 30 min, washed, and the fluorescence was quantitated by the Pandex.

Blocking of EGF Binding by Anti-EGFR MAbs. To determine if any of the anti-EGFR MAbs blocked EGF binding to the EGFR on intact cells, 200 μl of A431 cells (5×10^6 cells/ml) were mixed with 200 μl of various concentrations of either purified MAbs or unlabeled murine EGF (Sigma), in triplicate, starting at 6 μM, 1.0 mg/ml or 100 μg/ml, respectively, and incubated 30 min on ice. Murine ^{125}I-EGF, 6×10^4 cpm in 100 μl, was added to each sample and incubated an additional 30 min on ice. Cells were washed once, counted on th gamma counter, and percentage of bound counts was calculated and the average of triplicates plotted.

Immunofluorescence Staining of Cells. The cell lines A431 and the breast adenocarcinoma cell line SK-BR-3, previously shown to express amplified levels of p185^{HER2} (40, 41), were used to characterize the EGFR and p185^{HER2} specific panels of MAbs by FACS techniques. Confluent monolayers of cells were harvested with 25 mM EDTA in PBS, washed, and resuspended at 1×10^6 cells/ml in PBS containing 1% FBS (1% FBS/PBS). An aliquot, 1 ml, of each cell line was added to 10 μg of the appropriate MAb and incubated for 1 h on ice. The cells were washed twice with 1% FBS/PBS and incubated with FITC conjugated F(ab')$_2$ fragment of goat anti-mouse IgG specific antibody (Zymed, South San Francisco, CA), for 30 min on ice. The cells were washed twice and resuspended in 0.5 ml of 1% FBS/PBS and analyzed on an EPICS 753 (Coulter, Hialeah, FL) FACS using 300 mW of 488 nm argon laser light for excitation and measuring emitted light with a 525 nm narrow band pass filter.

Inhibition of A431 Growth by Anti-EGFR MAbs. A431 cells were plated into 35-mm culture dishes at 1.0×10^4 cells/dish. Either an unrelated, isotype control MAb 9F6 (anti-gp120 of human immunodeficiency virus), the anti-EGFR MAbs, or the anti-185^{HER2} MAb 4D5 were added on day 0 at a final concentration of 2.5 μg/ml (16.7 nM). After 7 days the cells were removed from the plates by trypsinization and the total cell number was determined by Coulter counter. The difference in growth of cells treated with the experimental and control MAbs is expressed as the ratio of their respective cell numbers. Each MAb was assayed in duplicate and the cell counts were averaged. As an additional positive control, EGF at 40 ng/ml (8 nM) was included; previous work has shown this to be growth inhibitory for A431 cells (42, 43).

RESULTS

ELISA Characterization of MAbs. Since these anti-EGFR and anti-p185^{HER2} MAbs could be used to develop important diagnostic reagents, a variety of immunological techniques were used to determine their reactivity and specificity. The ability of the purified MAbs to bind to immobilized WGA purified EGFR r p185^{HER2} was determined by ELISA. Relative activities of the antibodies at various concentrations, measured as $A_{492\ nm}$, were determined and the results at 1-μg/ml MAb concentrations are presented (Fig. 1). Four of the five anti-EGFR MAbs (5G3, 6C5, 13A9, and 19C5) were specific for the EGFR, with their reactivity with p185^{HER2} at background levels. In contrast, MAb 3G2 appeared to bind to both the EGFR and p185^{HER2}. Our MAb 13A9 had reactivity to EGFR equivalent to the well characterized anti-EGFR MAb 108 (generous gift of Joseph Schlessinger, Rorer Biotechnology, Inc.) described earlier (44). The anti-p185^{HER2} MAbs exhibited positive but var' le reactivity against immobilized p185^{HER2} in ELISA and none of these MAbs reacted with immobilized EGFR.

MAb Immunoprecipitation f ^{32}P Labeled Receptors. The specificity of the MAbs was also evaluated by utilizing radi immunoprecipitation on ^{32}P labeled proteins. Purified MAbs were reacted with [γ-^{32}P]ATP autophosphorylated EGFR and p185^{HER2} receptor preparations (Fig. 2). All the MAbs raised to A431 cells immunoprecipitated EGFR, with MAbs 13A9 and 108 being the most efficient. MAb 3G2 als precipitated a

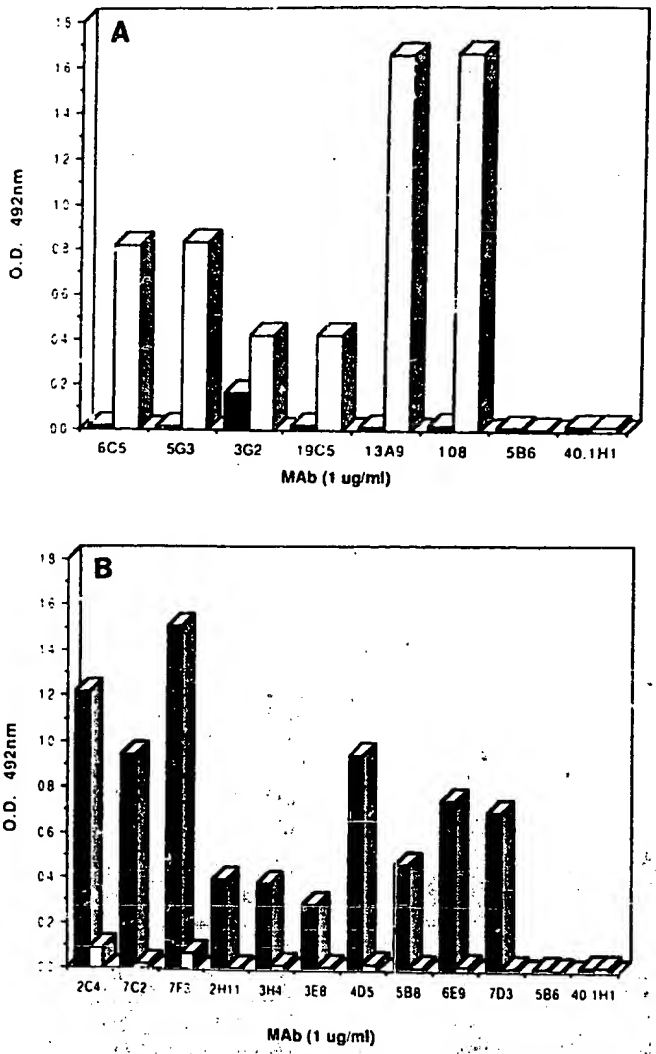


Fig. 1. Results from ELISA measuring the relative reactivities of either the purified anti-EGFR MAbs (*upper*) or the anti-p185^{HER2} MAbs (*lower*), at 1 μg/ml, with immobilized WGA affinity purified membrane extracts from A431 (□) and NIH 3T3/HER2-3$_{400}$ (■).

strong p185^{HER2} band by comparison to the EGFR band precipitated by this antibody (Figs. 2*A* and 3). These precipitation results are consistent with the ELISA results showing cross-reactivity of MAb 3G2 with both EGFR and p185^{HER2} coated plates. The MAbs against p185^{HER2} precipitated p185^{HER2} and not the EGFR (Fig. 2). None of the anti-p185^{HER2} MAb precipitations of the EGFR were above background levels of the negative MAbs 5B6 (anti-gp120 of human immunodeficiency virus) or 40.1.H1 (anti-human B surface antigen of hepatitis B virus), which gave some weak background bands due to slight overexposure of the autoradiography film prior to photographing. The MAbs specific for phosphotyrosine were also generated to investigate growth factor receptor structure and functi n including the associated tyrosine kinase activity. The anti-phosphotyrosine MAb 5E2 was pursued since it efficiently immunoprecipitated both the EGFR and p185^{HER2} (Fig. 3). MAb 5E2 did not react with either phosphoserine or phosphothreonine (data n t shown) and has also been useful in immunoblotting f phosphorylated EGFR receptors (45).

MAb Immunoprecipitation f [^{35}S]Cysteine Labeled Receptors. The antigen specificity of the MAbs was further confirmed by immun precipitation of the EGFR and p185^{HER2} from xtracts f A431 and SK-BR-3 cells metabolically labeled with [^{35}S]-

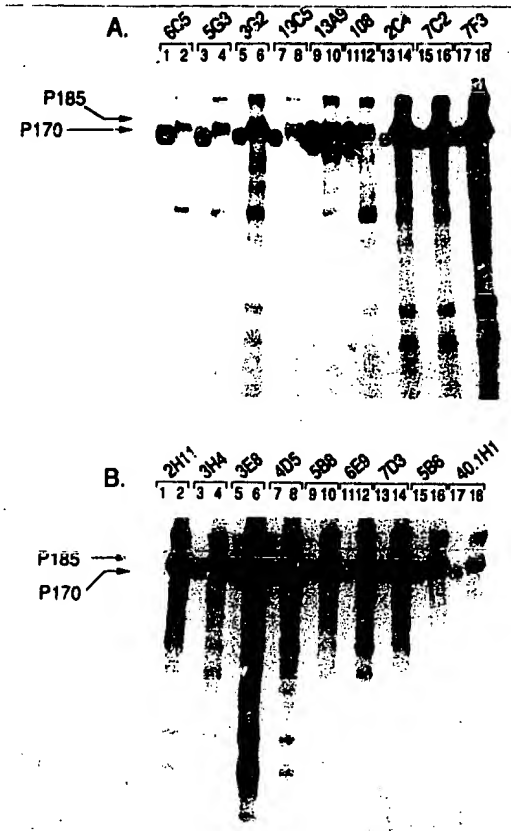


Fig. 2. Autoradiography of 7.5% SDS-polyacrylamide gel electrophoresis of [γ-^{32}P]ATP labeled proteins immunoprecipitated by either anti-EGFR MAbs (*A*, *Lanes 1–12*) or anti-p185^{HER2} MAbs (*A*, *Lanes 13–18*; *B*, *Lanes 1–14*) from either the A431 (*odd numbered lanes*) or the NIH 3T3/HER2-3$_{400}$ (*even numbered lanes*) WGA purified extracts. Negative control MAbs: 5B6 (anti-gp120 of human immunodeficiency virus, IgG$_1$; *Lanes 15* and *16*) and 40.1.H1 (anti-surface antigen B of hepatitis, IgG2a; *Lanes 17* and *18*) are included as isotype controls.

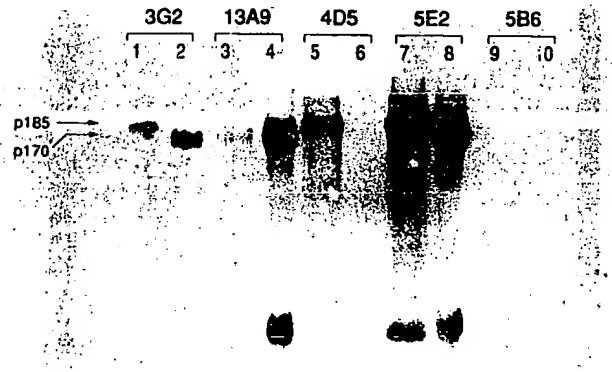


Fig. 3. Autoradiography of 7.5% SDS-polyacrylamide gel electrophoresis of [γ^{32}P]ATP autophosphorylated, labeled EGFR and p185^{HER2} comparing anti-EGFR, anti-p185^{HER2}, and anti-phosphotyrosine MAbs. The p185^{HER2} extracts are run in the *odd numbered lanes* and the EGFR extracts are run in the *even numbered lanes*. Anti-EGFR MAbs (*Lanes 1–4*), anti-p185^{HER2} MAb (*Lanes 5* and *6*) anti-phosphotyrosine MAb (*Lanes 7* and *8*) and control MAb (*Lanes 9* and *10*).

cysteine (Fig. 4). All the anti-EGFR MAbs precipitated the EGFR from the extracts of ^{35}S labeled A431 cells with n or few additional bands being precipitated from this heterogeneous extract of labeled cellular proteins by comparison to the negative controls (Fig. 4*A*). The MAbs 13A9 and 108 were the most efficient at immunoprecipitation and this trend is consistent with the immunoprecipitations using γ-^{32}P-labeled EGFR (Fig. 2*A*). In addition, MAbs 13A9, 108, and 5G3 als precipitated

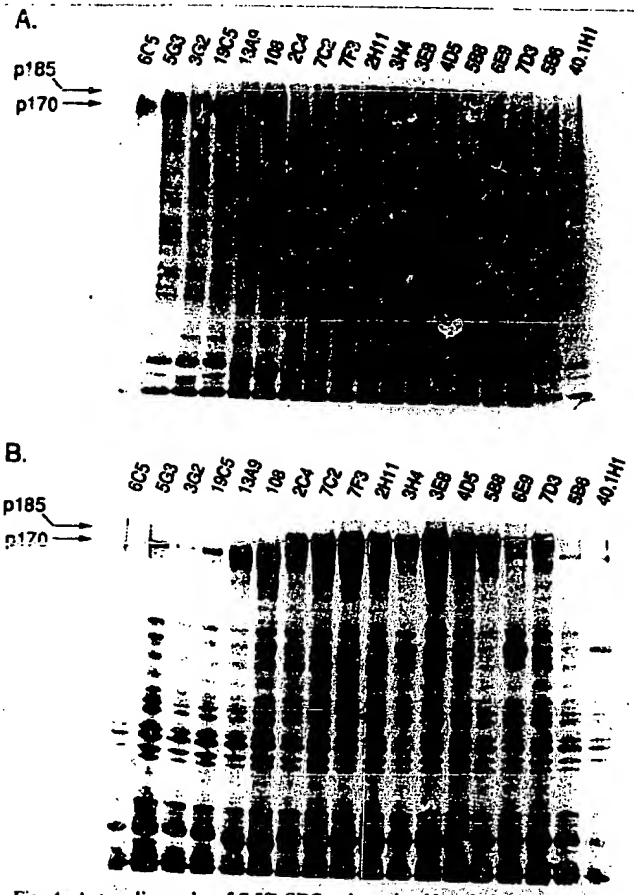


Fig. 4. Autoradiography of 7.5% SDS-polyacrylamide gel electrophoresis of [^{35}S]cysteine labeled proteins immunoprecipitated by anti-EGFR MAbs or anti-p185^{HER2} MAbs using (*A*) A431 cells and (*B*) SK-BR-3 cells.

the EGFR from the extract from SK-BR-3 cells (Fig. 4*B*). None of these EGFR MAbs, including MAb 3G2, precipitated p185^{HER2} from the [^{35}S]cysteine labeled SK-BR-3 extracts. The lack of detectable p185^{HER2} in the immunoprecipitation of [^{35}S]cysteine labeled p185^{HER2} by the anti-EGFR MAb 3G2 may be due to the relative lower specific activity of the ^{35}S *versus* ^{32}P labeled receptors.

The anti-p185^{HER2} MAbs 3E8 and 7F3 were the most efficient in precipitating [^{35}S]cysteine labeled p185^{HER2}; 2C4, 2H11, 4D5, 5B8, 7C2, and 7D3 were intermediate, while 3H4 and 6E9 were the least efficient (Fig. 4*B*). None of the p185^{HER2} MAbs immunoprecipitated the EGFR. These data are consistent with the rank order observed in the immunoprecipitations with autophosphorylated p185^{HER2}.

Mab Immunoprecipitation of Native and Deglycosylated Receptors. To determine whether individual MAb binding epitopes are protein or carbohydrate in composition, immunoprecipitations were performed on ^{32}P labeled, tunicamycin treated, and native EGFR from A431 cells and p185^{HER2} from NIH 3T3/HER2-3$_{400}$. The autoradiography bands from the MAb immunoprecipitations were compared with those from appropriate polyclonal antisera, following the rationale that those antisera bound both glycosylated and unglycosylated receptors. Anti-EGFR MAbs 3G2, 5G3, 6C5, 13A9, 19C5, and 108 immunoprecipitated both the native (M_r 170,000) and the l wer molecular weight (approximately M_r 140,000), deglycosylated forms of the EGFR (Fig. 5*A*). The p140 band appears only in the tunicamycin treated A431 cells and n t in the untreated cells prepared by the same meth d. The anti-hepatitis MAb 40.1H1 reproducibly bound to a deglycosylated determinant on the

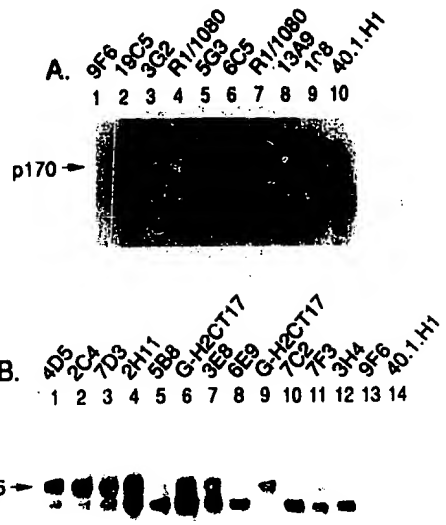


Fig. 5. Autoradiography of tunicamycin treated, partially nonglycosylated, [γ-^{32}P]ATP labeled A431 proteins immunoprecipitated by the anti-EGFR MAbs (*A*) or NIH 3T3/HER2-3$_{400}$ proteins immunoprecipitated by anti-p185^{HER2} MAbs (*B*). In *A*, *Lanes 2–3*, *5–6*, and *8–9* are immunoprecipitations with anti-EGFR MAbs; *Lanes 4* and *7* are immunoprecipitations with unglycosylated and native proteins, respectively, with mouse antiserum to the EGFR and *Lanes 1* and *10* are negative MAb immunoprecipitations with 9F6 (anti-gp120 of human immunodeficiency virus) and 40.1.H1 (anti-surface antigen B of hepatitis virus) respectively, with partially deglycosylated proteins. In *B*, *Lanes 1–5*, *7*, *8*, and *10–12* are immunoprecipitations with anti-p185^{HER2} MAbs; *Lanes 6* and *9* are immunoprecipitations with a mixture of deglycosylated and native proteins, respectively, with antiserum, G-H2CT17, to the COOH terminus of p185^{HER2} and *Lanes 13* and *14* are negative MAb immunoprecipitations as in *A*.

EGFR. This cross-reactivity was not seen with the anti-gp120 MAb 9F6. The anti-p185^{HER2} MAbs 3H4, 5B8, 6E9, 7C2, and 7F3 bound preferentially to the lower molecule weight, deglycosylated p185^{HER2} (approximately M_r 170,000), whereas 2C4, 2H11, 4D5, and 7D3 bound preferentially to the glycosylated form of p185^{HER2}, and 3E8 bound equally to the native and deglycosylated forms of p185^{HER2} by comparison to the polyclonal antisera G-H2C17 (Fig. 5*B*). As with the deglycosylated EGFR, the lower molecular weight band appears only in the tunicamycin treated NIH 3T3/HER2-3$_{400}$ cells and not in the untreated cells prepared by the same method. The immunological characteristics of the MAbs described including isotype, immunogen, ELISA reactivity, immunoprecipitation results, and epitope analysis and composition are summarized in Table 1.

Anti-EGFR MAbs Blocking of EGF Binding. The anti-EGFR MAbs were evaluated for their ability to block the ligand binding to the EGFR on A431 cells (Fig. 6). The anti-EGFR MAbs 5G3 and 6C5 inhibited ^{125}I-murine EGF ligand binding to receptor on A431 cells by 40–50% at an IgG concentration of 0.6 μM. MAb 19C5 showed 40% inhibition of ^{125}I-EGF binding at 6.0 μM, while MAb 108 inhibited ^{125}I-EGF binding by 25% to an IgG concentration of 0.06 μM. MAb 13A9 did not inhibit even at 6 μM, but instead appeared to enhance iodo-EGF binding. The unlabeled murine EGF control blocked ^{125}I-EGF binding by 95% at 6.0 and 0.6 μM.

FACS Analysis of MAbs Binding to Cells. To determine whether the MAbs could bind to and distinguish EGFR and p185^{HER2} on the surface of viable cells, FACS analysis was used with A431 and SK-BR-3 cells and the RMF of the molecules bound was calculated for each MAb (Fig. 7). The anti-EGFR MAbs bound to A431 cells with RMF values at least 100-fold greater than those measured on SK-BR-3 cells. This trend is consistent with the EGFR immunoprecipitation results from A431 and SK-BR-3 with the anti-EGFR MAbs (Fig. 4*B*). Although weak, all the EGFR MAbs, except 13A9, bound to the EGFR present on SK-BR-3 cells. The inconsistency with anti-EGFR MAb 13A9 in immunoprecipitation and FACS analysis may reflect an EGFR epitope which is sequestered on intact A431 cells. With the exception of MAbs 5B8 and 6E9, the anti-p185^{HER2} MAbs bound to SK-BR-3 cell with RMF values ranging 10- to 100-fold greater than the A431 cell values. These results are consistent with observed EGFR copy numbers of 1.2×10^6 and 8.7×10^4 for A431 and SK-BR-3 cells, respectively,[3] and a relative p185^{HER2} expression of approximately 20-fold lower on A431 and 10-fold higher on SK-BR-3 cells, by comparison to the EGFR expression on these cell lines.[4] The NIH 3T3/HER2-3$_{400}$ cells express 1.0×10^5 copies of p185^{HER2}. We have not determined the number of murine EGFR expressed by NIH 3T3/HER2-3$_{400}$ cells. Fig. 3 demonstrates that the anti-EGFR MAb 13A9 immunoprecipitates a weak p170 band from this cell line. Until we determine whether the entire anti-EGFR MAb panel binds to murine EGFR, the specificity of either the EGFR or p185^{HER2} specific MAbs should be concluded from the reactivity data generated with the human A431 and SK-BR-3 cell lines.

Growth Inhibitory Effects of Anti-EGFR MAbs on A431 Cells. We also investigated whether any of the anti-EGFR MAbs had growth inhibitory effects on A431 cells *in vitro*. Anti-EGFR MAbs 5G3 and 6C5, at final concentration of 16.7 nM, inhibited A431 cell proliferation by approximately 85% when compared to the A431 cell proliferation in the presence of anti-p185^{HER2} MAb 4D5 control (Fig. 8). This level of growth inhibition was equivalent to that of the EGF control at 8 nM. Anti-EGFR MAbs 13A9, 108, 3G2, and 19C5 had A431 growth inhibitory effects ranging from 50 to 30%, respectively. Hudziak *et al.* (38) performed similar *in vitro* growth inhibition experiments with the anti-p185^{HER2} MAbs and demonstrated that 4D5 was the most effective growth inhibitor of SK-BR-3.

DISCUSSION

We have used the established human epidermoid tumor line, A431, and a stably transfected murine cell line expressing the HER2/*neu* gene, NIH-3T3/HER2-3$_{400}$, as immunogens to produce MAbs which bind to the EGFR or p185^{HER2} on the surface of human cells. The resultant MAbs were used to investigate the expression and compare antigenic determinants on the extracellular domains of the EGFR and the related p185^{HER2}. Several groups have produced EGFR specific MAbs [(43, 46–49) partial list], some of which recognize noncarbohydrate determinants and react with the EGFR on a variety of cells. However, there has been little published antigenic characterization of these antibodies with respect to their cross-reactivity with p185^{HER2}. Schechter *et al.* (50) has shown that rabbit antisera raised to EGFR from A431 cells will immunoprecipitate p185^{neu} from NIH 3T3 cells transfected with the rat, ethylnitrosourea-induced *neu* oncogene. In contrast, the anti-rat p185^{neu} MAb 7.16.4 described by Drebin *et al.* (51) does not react with either the human p185^{HER2} or the human EGFR (50). The previous description of p185^{HER2} specific MAbs, reported by van de Vijver *et al.* (52), did not address the question of cross-reactive determinants present on both the EGFR and p185^{HER2}. Results from immunoprecipitation experiments with immune perfusion sera (data not shown) from our BALB/c

[3] M. Winget, personal communication.
[4] G. D. Lewis, personal communication.

Table 1 *Summary table of monoclonal antibodies described*

MAb	Isotype	EGFR ELISA[a]	p185^{HER2}ELISA	EGFR RIP	p185^{HER2}RIP	Epitope	FACS
3G2	IgG1κ	+	+	+	+	A(p)	+++
5G3	IgG1κ	++	−	++	−	B(p)	+++
6C5	IgG1κ	++	−	++	−	B(p)	+++
13A9	IgG1κ	+++	−	+++	−	C(p)	+++
19C5	IgG1κ	+	−	+	−	D(p)	+++
108[b]	IgG2aκ	+++	−	+++	−	E(p)	+++
2C4	IgG1κ	−	+++	−	++	F(c)	+++
2H11	IgG2aκ	−	+	−	++	H(c)	++
3E8	IgG2aκ	−	+	−	+++	H(p)	+++
3H4	IgG1κ	−	+	−	+	I(p)	+
4D5	IgG1κ	−	++	−	++	I(c)	+++
5B8	IgG1κ	−	+	−	++	nd(p)[c]	+
6E9	IgG1κ	−	++	−	+	nd(p)	−
7C2	IgG1κ	−	++	−	++	G(p)	+++
7D3	IgG1κ	−	++	−	++	F(c)	+++
7F3	IgG1κ	−	+++	−	+++	G/F(p)	+++
5E2	IgG1κ	nd	nd	+++	+++	nd	nd

[a] ELISA data columns (summary of $A_{492\ nm}$): <0.1 = −; 0.11–0.50 = +; 0.51–1.0 = ++; >1.0 = +++. RIP data columns (summary of autoradiography from immmunoprecipitations): bands equal to negative control = −; weak bands but darker than negative control = +; moderately exposed bands = ++; strongly exposed bands = +++. Epitope data columns: letters assigned to represent individual epitopes A through I. MAbs were considered to share an epitope if each blocked binding of the other by 50% or greater in comparison to an irrelevant MAb control. The epitope composition recognized by immunoprecipitations with each MAb from tunicamycin treated cells is shown. A MAb is interpreted to bind predominantly to either a (p) protein determinant if that MAb binds preferentially to deglycosylated or equally to both the native and deglycosylated forms of the EGFR or p185^{HER2} or (c) carbohydrate determinant if that MAb binds preferentially to the native forms of the EGFR or p185^{HER2}. FACS data column: fluorescence staining of either A431 cells by the anti-EGFR MAbs or SK-BR-3 cells by the anti-p185^{HER2} MAbs equal to the negative control MAbs = −; 1–9-fold higher than the negative controls = +; 10–99-fold higher than the negative controls = ++; >100-fold higher than the negative controls = +++.

[b] Generous gift from J. Schlessinger.

[c] nd, not done.

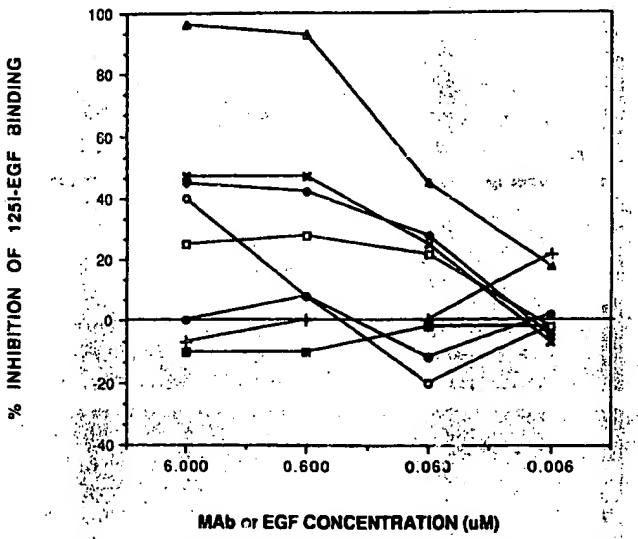
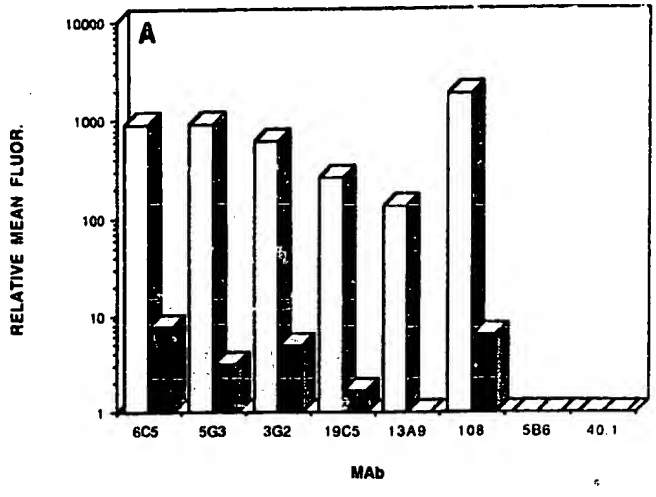


Fig. 6. MAb blocking of the ^{125}I-murine EGF binding to A431 cells. Presented are the dose-response curves with purified anti-EGFR MAbs 6C5 (×), 5G3 (◆), 3G2 (+), 19C5 (○), 13A9 (■), 108 (□) compared to EGF (▲) and anti-phosphotyrosine MAb, 5E2 (●). The results are plotted as percentage of inhibition compared to maximal ^{125}I-EGF binding in buffer.

mice immunized with either A431 or NIH 3T3/HER2-3$_{400}$ cells showed no detectable antibodies which recognize shared, immunodominant epitopes on preparations of EGFR or p185^{HER2}. The resulting hybridomas produced from these mice were evaluated for antibody binding specificity to the EGFR or p185^{HER2} using either soluble receptors or intact cells. The specificity of these MAbs for Triton X-100 solubilized receptors was determined by ELISA on immobilized WGA purified membrane extracts and by immunoprecipitation using either extracts of [^{35}S]cysteine metabolically labeled cells or [γ-^{32}P]ATP autophosphorylated membrane extracts. Reactivity with extracellular domains of the receptors was determined by immunofluorescence by using the Pandex screen machine technology and FACS analysis on intact, viable cells. We have shown that our five anti-EGFR MAbs define at least three epitopes on the extracellular domain of the EGFR. One of these epitopes is shared by p185^{HER2} and is recognized by MAb 3G2. The p185^{HER2} MAbs recognize at least four epitopes on the extracellular domain of p185^{HER2} and none of these epitopes are shared with the EGFR. Experiments with the truncated, extracellular domain of the p185^{HER2} molecule (data not shown) indicates anti-p185^{HER2} MAbs 5B8 and 6E9 bind to epitopes very close to the transmembrane domain and may not be accessible to antibody binding on the surface of intact SK-BR-3 cells as compared to soluble p185^{HER2} receptors.

The binding site recognized by the individual MAbs was evaluated for its protein or carbohydrate composition by tunicamycin inhibition of glycosylation. The five EGFR specific MAbs bind preferentially to the protein backbone of the EGFR. This is in contrast to many anti-EGFR MAbs reported that bind to carbohydrate blood group determinants found on the EGFR of A431 (47, 48) which are not expressed on th EGFR from other human cells. We have shown that our highest affinity anti-EGFR MAbs immunoprecipitate the EGFR from both A431 and SK-BR-3 cells. By comparison, 5 of the 10 anti-p185^{HER2} MAbs bind preferentially to the protein backbone while the remaining 5 MAbs bind either selectively to the glycosylated receptor or equally to both forms. The significance of the epitope composition recognized by the anti-p185^{HER2} MAbs may be elucidated as we continue to characterize the tissue distribution and epitope expression of p185^{HER2} in various tumors and normal tissues.

Hudziak *et al.* (38) has shown that several of the anti-p185^{HER2} MAbs inhibit breast tumor cell line proliferation *in vitro*. In an effort to perform similar studies on the EGFR specific MAbs, we determined the growth inhibitory effects of the anti-EGFR MAbs on the A431 cell line. MAbs 5G3 and 6C5 showed the greatest inhibition (85%) of cell proliferation. The cell proliferation data are consistent with the results obtained when the anti-EGFR MAbs were tested for blocking of murine ^{125}I-EGF binding to receptor on intact A431 cells. MAbs 5G3 and 6C5 demonstrated the strongest blocking of



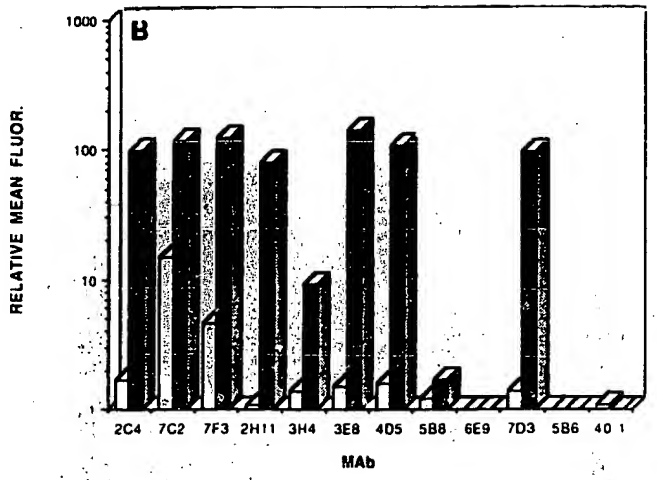


Fig. 7. RMF binding of purified anti-EGFR MAbs (*upper*) or the anti-p185HER2 MAbs (*lower*) to A431 (□) and SK-BR-3 (■). Included are the negative isotype controls 5B6 (IgG1) and 40.1.H1 (IgG2a). RMF was calculated as

$$N = e^{(0.693/x)C}$$

where N is the "linearized fluorescence value", x is the log to linear transforming factor, and C is the channel number.

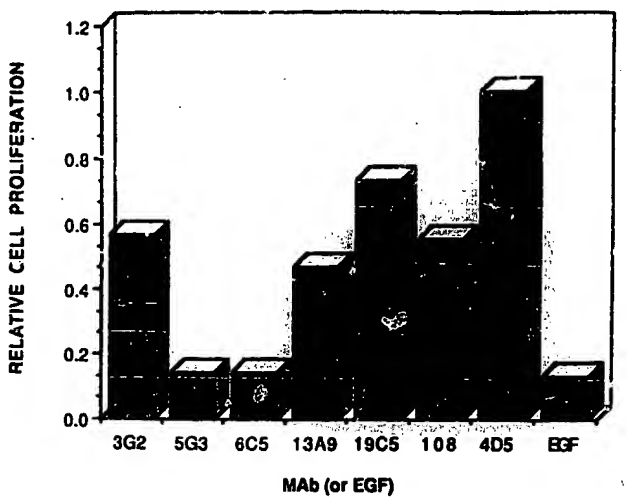


Fig. 8. The total number of A431 cells, after a 7 day growth period with either MAb at 2.5 μg/ml or EGF at 40 ng/ml, is compared using the anti-EGFR MAbs and anti-p185HER2 MAb 4D5. The relative proliferation is calculated by dividing the total A431 cells in the presence of anti-EGFR MAbs by the total A431 cells in the presence of anti-p185HER2 MAb 4D5.

the EGF binding (45% inhibition), while 19C5 and 108 show weaker EGF blocking (25–40% inhibition), and 13A9 and 3G2 did not block murine EGF binding to A431 cells. These results could reflect the mechanism by which MAbs to the EGFR inhibit A431 cell division *in vitro*. Similar receptor blocking experiments with the anti-p185HER2 MAbs await identification of the putative ligand.

It appears that in mice the major immunodominant determinants on the EGFR and p185HER2 are not in the homologous regions comprising 40% of the extracellular domains of these receptors. It has previously been shown that a membrane structure sharing homologous extracellular regions to the EGFR and p185HER2 exists in *Drosophila* (53, 54). A high degree of conservation throughout evolution, in certain extracellular regions of the EGFR and p185HER2, could be accompanied by a lack of immunogenicity in these regions. Since two of the EGFR specific MAbs described here partially block EGF binding, it is likely that at least one of the immunogenic, nonhomologous epitopes on the EGFR is located in or near the ligand binding site. The possibility does exist that we have broken immunological tolerance to the murine EGFR, and future experiments, pending identification of murine cell line(s) which overexpress the EGFR, will include evaluation of these EGFR MAbs for species specificity. We have observed by FACS analysis (data not shown) that our p185HER2 MAbs do not bind to the endogenous *neu* on the surface of RAT-1 cells indicating that, like MAb 7.16.4, they bind to p185HER2 epitopes that may be species specific. This result might be predicted since the anti-p185HER2 MAbs resulted from fusions in the murine system and mice may be tolerant to conserved determinants on p185HER2. Whether the immunogenic, nonhomologous regions on p185HER2 are in the putative ligand binding pocket will require further investigation.

Overexpression or altered forms of the EGFR and/or p185HER2 are found in many forms of human cancers including cervical, ovarian, squamous, adenocarcinomas, and primary breast tumors. In the case of breast and ovarian cancer, amplification of HER2/*neu* expression is associated with approximately 30% of the primary tumors tested and is correlated with metastatic disease and poor prognosis (17, 18). The location of these receptors on the surface of cells, prognostic significance and probable causal role makes them very attractive targets for production of monoclonal antibodies for immunodiagnostics and immunotherapy. Immunohistochemistry data on tumor biopsy tissue could be useful in determining the aggressiveness of therapy depending on the level of p185HER2 expression. These MAbs would also be candidates for *in vivo* radioimaging for detection of relevant primary tumors and micrometastases. The receptor nature of these antigens, including surface location, ligand binding, and receptor/ligand complex internalization, makes tumors expressing these receptors potential targets for immunotherapeutic intervention utilizing antibodies as radioconjugates, drug carriers, or immunotoxins. An alternate approach would be to exploit the ligand blocking characteristics of some receptor specific MAbs and deprive transformed cells of their intracellular signal transduction. This has been demonstrated with EGFR specific MAbs where EGF blocking is the mechanism for growth inhibition on EGF dependent cells (43, 55). The anti-p185HER2 MAbs should be useful reagents to investigate similar receptor/ligand studies on p185HER2 and may facilitate identification of the putative ligand.

We are optimistic that diagnostic and therapeutic applica-

tions of MAbs specific for receptor related molecules associated with human malignancy will allow exploitation of this probable causal relationship in transformation and the process of oncogenesis.

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#350 Wednesday, April 24, 1996, 1:00–5:00, Poster Section 4

Reduced concentrations of signal transducing effector molecules in multistage lung carcinogenesis in mice. Pamela L. Rice, Lori D. Dwyer-Nield, Anita C. K. Miller, Andrius Kazlauskas and Al Malkinson. *University of Colorado Health Sciences Center, School of Pharmacy, Molecular and Environmental Toxicology Program, University of Colorado Cancer Center, and National Jewish Center for Respiratory Immunology, Denver, CO.*

EGF and PDGF bind to their respective receptors to elicit a series of tyrosine phosphorylation events that recruit signal transduction molecules to the receptors, activate ras, and elevate transcription of growth-related genes. Altered signal transducing pathways are a hallmark of carcinogenesis. A mutation in K-ras is the initiating lesion in mouse lung tumorigenesis. Decreased concentrations, as assessed by immunoblotting, of the following were found in benign and malignant lung tumors: EGF-R, PDGFR-α, ras-GAP, protein kinase C-α, and calpain II. Studies performed on cell lines isolated from mouse lung epithelium correspond to these *in vivo* results. The non-tumorigenic line, E10, contained more of each of these same signal transducing molecules than its spontaneous transformant, E9. This supports the theory that as cells become transformed they lose the ability to respond to extracellular regulatory signals. (Supported by ES02370 and CA33497.)

#351 Wednesday, April 24, 1996, 04:10–04:25, Room 31

Environmental stresses signal apoptosis through a ceramide-initiated sapk/jnk-mediated cascade. M. Verheij, R. Bose, X.H. Lin, B. Yao, W.D. Jarvis, S. Grant, M.J. Birrer, E. Szabo, L. I. Zon, J. M. Kyriakis, A. Haimovitz-Friedman, Z. Fuks and R.N. Kolesnick, *Department of Radiation Oncology and Laboratory of Signal Transduction, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.*

Recent evidence suggests that tumor necrosis factor a (TNFa), Fas and ionizing radiation signal through the sphingomyelin pathway to induce apoptosis. The sphingomyelin pathway is initiated by hydrolysis of plasma membrane sphingomyelin to generate ceramide by the action of a sphingomyelinase. Ceramide serves as a second messenger stimulating a cascade of kinases and transcription factors that activate a final common pathway of programmed cell death. We have tested a variety of cellular stress factors for their effect on ceramide levels and subsequent induction of apoptosis. U937 human monoblastic leukemia and bovine aortic endothelial cells were treated with ionizing radiation (10 Gy), ultraviolet radiation C (10,000 mJ/cm^2), H_2O_2 (1 mM), heat shock (45°C) or TNFa (10 nM). This panel of stresses produced within seconds to minutes significant ($p < 0.05$) elevations of cellular ceramide levels in a dose-dependent manner. Ceramide remained elevated for at least 30 minutes and there was a concomitant quantitative reduction in cellular sphingomyelin content. Apoptotic DNA degradation and typical morphological changes of apoptosis appeared as soon as 6 hours after stimulation and were time- and dose-dependent. Apoptosis was specific for ceramide as the cell-permeable synthetic ceramide analog C2-ceramide mimicked this effect, whereas synthetic analogs of other potential lipid second messengers were ineffective in inducing an apoptotic response. These findings suggest that a variety of extracellular stress agents employ a common signaling pathway to trigger apoptosis in both proliferating and non-proliferating cells.

#352 Wednesday, April 24, 1996, 1:00–5:00, Poster Section 4

Phospholipase C β_1 in the nucleus: Its key role in cell division. Lucio Cocco, R. Stewart Gilmour* and Francesco A. Manzoli *Institute of Anatomy, University of Bologna, Italy and AFRC Babraham Institute, Cambridge, England**.

Besides the membrane localisation also the nucleus shows to be site for both synthesis and hydrolysis of the phosphorylated forms of phosphatidylinositol. Previous observations have assigned a role to PLC β_1 in IGF-I stimulated cell growth in that the nuclear PLC β_1 is rapidly and transiently activated inducing increased diacylglycerol generation and translocation of protein kinase C α to the nucleus. Using Swiss 3T3 cells stably transfected with an antisense PLC β_1 construct the knock-out of the PLC β_1 gene induces both a loss of the nuclear enzyme, as determined by Western Blots and a loss of the mitogenic responsiveness to IGF-I. On the contrary, overexpression of nuclear PLC β_1 in stably transfected Swiss 3T3 cells increases dramatically both the phospholipase C activity and the mitogenic responsiveness to IGF-I. All in all these data show a direct relationship between nuclear PLC β_1 evoked signals and IGF-I induced cell growth and indicate PLC β_1 as a key element of the nuclear autonomous signalling leading to the onset of DNA synthesis induced by growth factors acting at the plasma membrane through tyrosine kinase receptors.

L.C. is supported by Italian CNR PF ACRO.

#353 Sunday, April 21, 1996, 1:00–5:00, Poster Section 12

Effects of the 4D5 antibody on HER2/neu heterodimerization with other class I receptors in human breast cancer cells. Reese D, Arboleda J, Twaddell J, Akita R*, Sliwkowski M*, Slamon D. *Division of Hematology/Oncology, UCLA School of Medicine, Los Angeles, CA, *Genentech, Inc. South San Francisco, CA.*

Overexpression of the HER2/neu oncogene correlates with a poor prognosis in human breast and ovarian cancer and likely plays a role in the pathogenesis of these diseases. To examine HER2 signal transduction, we studied the effects of the EGF and heregulin families of ligands as well as anti-HER2 antibodies on human breast cancer cells which overexpress HER2. Heregulin induces transient tyrosine phosphorylation of HER2, HER3, and HER4, while EGF and TGFα induce phosphorylation of EGFR, HER2, and HER3. The anti-HER2 antibodies 4D5 and 7C2 induce prolonged phosphorylation of HER2 only. In addition, HER2 forms receptor heterodimer pairs with EGFR, HER3, and HER4 as demonstrated by co-immunoprecipitation and receptor cross-linking studies. The anti-proliferative 4D5 antibody disrupts HER2 association with HER3 and HER4. These data support a model in which HER2 forms functional heterodimers with each of the other class I receptors; these interactions may be disrupted by the 4D5 antibody, which may have effects on downstream signalling pathways.

#354 Wednesday, April 24, 1996, 1:00–5:00, Poster Section 4

The differential effects of perillyl alcohol on untransformed D27 vs. k-*ras* transformed B12/13 pancreatic ductal epithelial cells. Stayrook, K.R., McKinzie, J.H., Ayoubi, A.S., and Crowell, P.L. *Dept. of Biology, Indiana Univ.-Purdue Univ. at Indianapolis, Indianapolis, IN 46202.*

Due to the importance of prenylation for the function of many proteins involved in mitogenic signalling, including oncogenic *Ras*, inhibitors of prenylation are an attractive source of drugs for treatment of cancer. The monoterpene perillyl alcohol is an inhibitor of prenyl protein transferases and inhibits the prenylation of 21–26kDa small G proteins in both D27 and B12/13 cells. Perillyl alcohol also inhibits the growth of D27 and B12/13 cell lines with IC_{50} values of 260 μM and 150 μM, respectively. However, this inhibition of protein prenylation and cell growth occurs to a greater extent in B12/13 cells than in D27 cells. B12/13 cells exhibit only 55% of control cell growth while D27 cells maintain 80% of control cell growth when treated with 100 μM perillyl alcohol. To assess whether inhibition of B12/13 cell growth is due to decreased function of *Ras*, we measured *Ras* farnesylation, *Ras* GTP/GDP ratios, and ERK1/2 activity. 30 μM lovastatin inhibited *Ras* farnesylation whereas 100–500 μM perillyl alcohol did not. Similarly, 30 μM lovastatin decreased the percentage of GTP-bound *Ras* to 10.3% as compared to 19.5% in the untreated control, but perillyl alcohol had no effect on the percentage of GTP-bound *Ras*. In contrast, neither perillyl alcohol nor lovastatin had any effect upon ERK1/2 activity in B12/13 cells. In conclusion, perillyl alcohol does inhibit protein prenylation in pancreatic cells but it seems that *Ras* farnesylation is not affected by the drug. Differential effects of perillyl alcohol in this pair of cell lines may be due instead to perillyl alcohol-induced apoptosis, since perillyl alcohol increases apoptosis 8-fold in pancreatic carcinoma cells. Support: NIHCA64297.

#355 Wednesday, April 24, 1996, 1:00–5:00, Poster Section 4

Expression of PKC isoforms in B16F10 tumor cells in vitro and in vivo. Szalay, J., Bhati, R., Bruno, P., Pathak, S., †Rotenberg, S., Ganjian, P., *Depts. Biology & †Chemistry, Queens College, Kissena Blvd, Flushing NY 11367*

Immunocytochemistry was used to examine the expression and cellular localization of nine PKC isoforms in cultured B16F10 melanoma cells, subcutaneous (SC) tumors and lung metastases. Using at least 8 assays/isoform, the percent of samples showing positive expression of each isoform was determined. Cultured cells expressed PKC alpha, beta 1, beta 2, delta, gamma, epsilon, eta, theta and zeta in 75–100% of the assays performed. Some isoforms appeared to be uniformly dispersed throughout the cytoplasm, others appeared to be concentrated in peri- or intra-nuclear locations, in 1–2 discrete areas immediately adjacent to the nucleus, or within elongated cytoplasmic processes. In SC tumors PKC alpha, beta 1, beta 2, gamma, delta, epsilon, eta and zeta were expressed in 50–93% of the assays, while theta was expressed in only 33% of the assays. Most lung metastases showed little or no expression of PKC isoforms. Alpha, beta 1, zeta, epsilon and theta were expressed in 22–45% of the assays, and expression of gamma, delta, eta, and theta was not observed. PKC isoform expression was also examined using immunoblotting techniques, and the results compared with those obtained using immunocytochemistry. In summary, B16F10 melanoma cells are capable of expressing all PKC isoforms examined, but in vivo expression depends upon the location of the tumor and other unidentified parameters.

#356 Wednesday, April 24, 1996, 02:25–02:40, Room 31

Activation of protein kinase C (PKC) isozymes inhibits cell cycle progression, modulates phosphorylation of the retinoblastoma (Rb) protein, and induces expression of p21$^{waf1/cip1}$ in intestinal epithelial cells. Frey, M.R., Zhao, X., Evans, S.S., Black, J.D. *Roswell Park Cancer Institute, Buffalo, NY, 14263.*

Previous studies in this laboratory support a role for PKC isozymes in control of post-mitotic events in the intestinal epithelium *in situ* (J. Cell Biol. 126, 747–763, 1994). Using the IEC-18 immature intestinal epithelial cell line as a model system, we have explored the role of PKC isozymes in control of intestinal epithelial cell growth and cell cycle progression. In these cells, treatment with phorbol ester resulted in activation of PKC α, δ and ε, concomitant with transient inhibition of cell cycle progression in G_1 and G_2/M, as measured by flow cytometry. Reversal of cell cycle arrest was coincident with downregulation of PKC α and δ. Treatment of IEC-18 cells with dioctanoyl-1,2-*sn*-glycerol, which does not downregulate PKC α and δ under the conditions used, produced a *sustained* inhibition of cell cycle progression. To explore the molecular pathways by which PKC isoforms interact with and modulate IEC-18 cell cycle control mechanisms, the effects of PKC activation on Rb protein phosphorylation/activity and p21$^{waf1/cip1}$ expression in IEC-18 cells were determined. PKC activation resulted in the appearance of the underphosphorylated, growth-suppressive form of Rb, and in the rapid accumulation of the cyclin-dependent kinase inhibitor p21$^{waf1/cip1}$. Maintenance of these effects coincided with the presence of active PKC α and δ in the cells. Taken